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PRINCIPAL INVESTIGATOR: Sreenivasa R. Chinni, Ph.D

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI 48202

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Sreenivasa R. Chinni, Ph.D

E-mail: schinni@med.wayne.edu

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14. ABSTRACT

Chemokines and chemokine receptor interactions facilitate the physiological migration of cells. Interaction of chemokines with their receptors leads to the expression/activation of adhesion molecules and proteases. Recent evidence suggests that a cancer cells utilize similar mechanism to metastasize to target tissues for secondary growth. Herein, we hypothesize that SDF-1 α (also known as CXCL12) and CXCR4 axis is active in PC cells and CXCL12 and CXCR4 interactions facilitates the metastasis of prostate cancer cells by activating intracellular signaling pathways leading to the expression and release of MMP-9. Using a variety of methods including RT-PCR, ELISA, gelatin zymography, cellular motility and invasion and sub-cellular fractionation of prostate cancer cells, we showed that (a) CXCL12 and CXCR4 axis is active in PC bone metastasis in inducing MMP-9 expression; (b) pharmacological inhibition of PI3 kinase and MAP kinase pathways abrogated the CXCL12 induced invasion of PC-3 cells; (c) CXCL12 induced Akt1 phosphorylation is indispensable for proMMP-9 secretion, migration and invasion of PC-3 cells; (d) CXCR4 over-expression in PC-3 cells induced expression of several members of proteases and chemokines in PC-3 cells and, (e) CXCR4 was localized to lipid raft fractions in PC cells. This data suggests that chemoattractive mechanisms may involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent release of MMPs into the local environment.

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CXCR4, SDF-1α, MMP and prostate cancer bone metastasis

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INTRODUCTION:

Prostate cancer cells frequently metastasize to bone. The bone-associated chemokines may play a role in the chemoattraction of prostate cancer cells to bone. The interaction of bone associated chemokines with the chemokine receptors on prostate cancer cells leads to the activation of signaling pathways and subsequent expression of proteases including MMP-9. The literature suggests the chemokine, CXCL12 formerly known as SDF-1α, and its cognate receptor CXCR4 are involved in the metastasis of breast cancer cell to lymph node and lung(1), further several recent studies show the existence these chemokine and chemokine receptor interactions in several types of cancers including, prostate(2, 3), ovarian(4), melanoma(5), colon(6), pancreatic(7, 8) and glioblastoma (3, 9) tumors. The purpose of current proposal is to demonstrate the presence of functional chemokine receptor; CXCR4 in PC cells and clinical PC bone metastasis, and its ligand CXCL12 in bone tissue, and to test the hypothesis that interaction of bone associated CXCL12 with CXCR4 expressed on prostate cancer cells elicits intra cellular signaling events leading to the MMP-9 gene expression. The experiments in the current proposal were designed to provide link between the chemoattraction of cancer towards target environment mediated by the CXCL12 and CXCR4, and expression of proteases by activation of cell signaling pathways in the bone target environment.

BODY

Expression of CXCR4 and SDF-1α in prostate cancer cells and human fetal bone stromal cells.

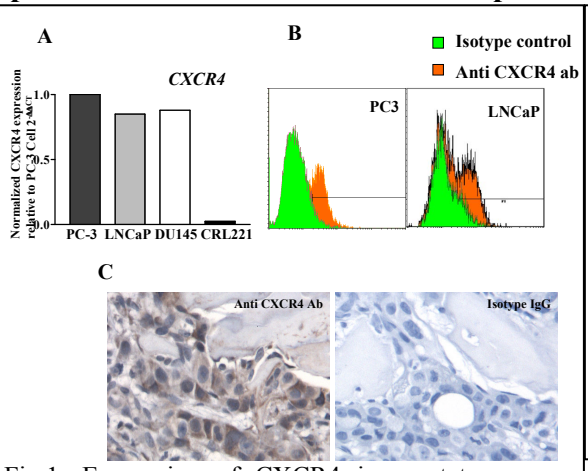

Fig.1. Expression of CXCR4 in prostate cancer cells. A. Real-time PCR expression of CXCR4 in prostate cancer cells and prostate epithelial cell line CRL2221. B. Fluorescence activated cell-sorting analysis of CXCR4 expression in PC-3 and LNCaP cells, C. Immuno histochemical analysis of CXCR4 in 6-day PC-3 SCID-human bone tumors.

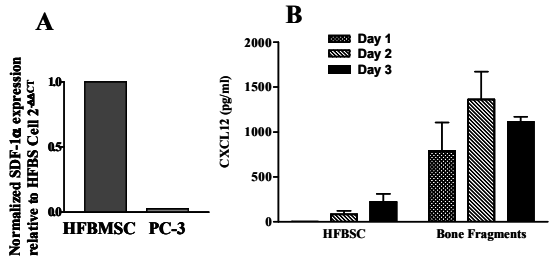

Fig. 2. Expression of SDF-1α in human fetal bone stromal cells and human fetal bone tissue. A. RT-PCR analysis of SDF-1 in *in vitro* cultures of human fetal bone stromal cells and PC-3 cells. B. ELISA assay of SDF-1α in the conditioned media of human fetal bone stromal cells and human fetal bone tissue.

The expression of CXCR4 (Fig. 1) and SDF-1α (Fig. 2) in prostate cancer cells and human fetal bone stromal cells were investigated with variety of techniques. RT-PCR analysis show that cancer cells express CXCR4 receptor and virus transformed prostate epithelial cells express very low levels of CXCR4 receptor (Fig.1 A). The chemokine SDF-1α is expressed on the primary cultures of human fetal bone stromal cells and PC-3 cells express very low levels (Fig. 2A). Fluorescence activated cell-sorting analysis of PC-3 and LNCaP cells show the cell surface expression of CXCR4 protein in both cell lines (Fig1 B). SCID-human PC-3 bone tumor tissue stained positive for CXCR4 expression (Fig1C). Human fetal bone stromal cell and human fetal bone tissue secreted SDF-1α protein were determined using SDF-1α specific ELISA, the results show that both cells and bone tissue secreted the chemokine SDF-1α and bone tissue secreted higher levels of SDF-1α (Fig2B).

SDF-1α induces MMP-9 expression in prostate cancer cells: MMP-9 gene expression in PC-3, LNCaP and DU145 cells was measured using the MMP-9 gene specific primers in RT-PCR experiment. Both PC-3 and LNCaP cells induced the MMP-9 gene expression. DU145 cells are not responsive to SDF-1α in MMP-9 gene expression (Fig. 2A). SDF-1α induced MMP-9 gene expression is further confirmed by MMP-9 promoter activation studies using the full length MMP-9 promoter element cloned in luciferase reporter vectors. PC-3 cells have a basal promoter activation

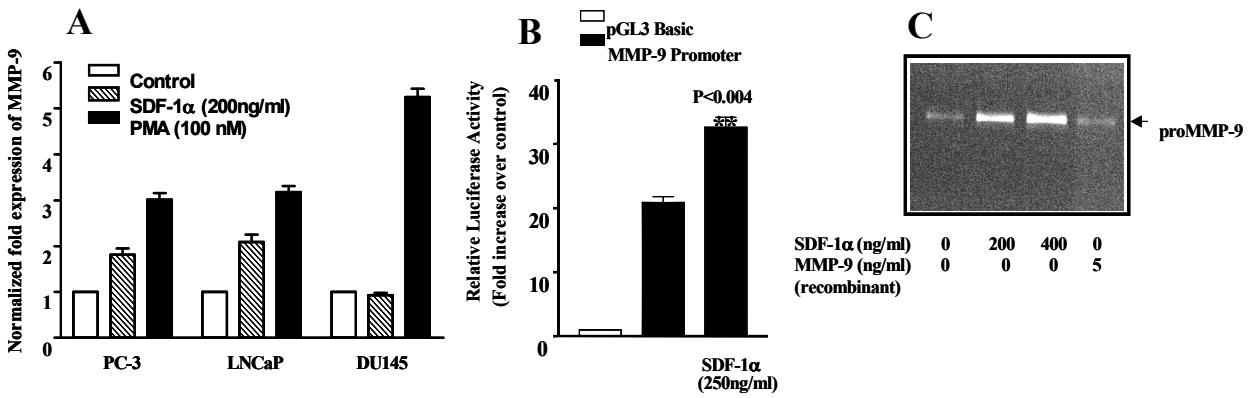

Fig.3. SDF-1α induces MMP-9 expression in prostate cancer cells. A. RT-PCR analysis of MMP-9 gene expression in prostate cancer cells. B. MMP-9 promoter activation. The PC-3 cells were transfected with MMP-9 promoter reporter construct and treated with SDF-1α and luciferase activity was measured. C. Gelatin zymogram analysis of conditioned media from PC-3 cells treated with SDF-1α.

and upon SDF-1α treatment there is a 10-12 fold more increase in MMP-9 promoter activity (Fig.2B). Further SDF-1α induced MMP-9 protein release from the PC-3 cells were evaluated by analyzing the conditioned media obtained from the PC-3 cells treated with different concentrations of SDF-1α. The results show that there is dose dependent increase in the proMMP-9 secretion from the cancer cells (Fig.2C).

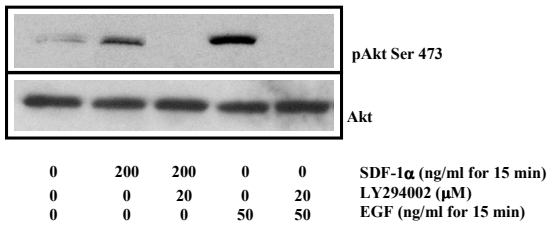

Fig. 4. SDF-1α activates Akt kinase in PC-3 cells. PC-3 cells were treated with indicated ligands in the figure and cell lysates were analyzed on the western blot with anti-Akt and anti-pAkt antibodies.

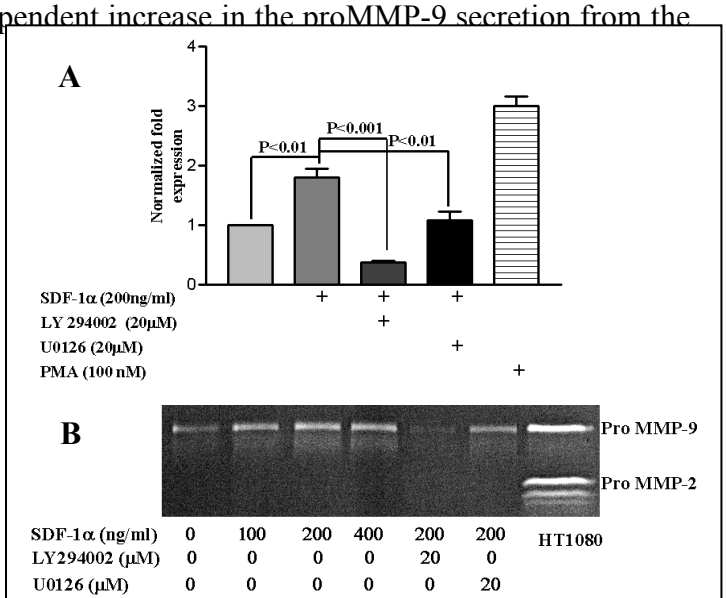

Fig. 5. SDF-1α induces MMP-9 expression in PC3 cells via PI3K and MAP kinase signaling pathways. A. RT-PCR analysis of MMP-9 gene expression in PC-3 cells. B. Gelatin zymogram analysis of conditioned media from PC-3 cells treated with indicated ligand and inhibitors.

SDF-1α induced signaling pathways participate in MMP-9 gene expression in PC-3 cells: SDF-1α induces PI3 kinase activation and subsequent phosphorylation of Akt in PC-3 cells. PC-3 cells were treated with SDF-1α and PI3 kinase inhibitor LY294002 and total cellular proteins were analyzed for Akt and activated form of Akt (pAkt). SDF-1α induced the phosphorylation of Akt at serine 473 position and PI3 kinase inhibitor LY294002

inhibited the SDF-1α induced Akt activation (Fig. 4). Further we evaluated the significance of Akt activation in MMP-9 gene expression PC-3 cells. PC-3 cells were treated with SDF-1α, PI3 kinase inhibitor LY294002 and MAP kinase inhibitor U0126 and PMA (positive control for MMP-9 gene expression in cancer cells) for 24 hours. MMP-9 gene expression is evaluated by RT-PCR (Fig. 5 A) and proMMP-9 protein secretion was measured by gelatin zymography (Fig. 5B). Both RT-PCR and zymography data suggest that SDF-1α induced MMP-9 gene expression in PC-3 cells and SDF-1α induced MMP-9 gene expression in PC-3 cells were inhibited by pretreatment of cells with both PI-3 kinase and MAP kinase inhibitors. PI3 kinase inhibition is more potent in inhibiting the MMP-9 gene expression and secretion from the PC-3 cells.

Bone cell-associated CXCL12 stimulates CXCR4-dependent migration of prostate cancer cells. To study the role of stromal cell-associated soluble CXCL12 in inducing chemomigration of PC-3 cells, we employed a transwell co-culture system where prostate cancer cells were cultured with either bone stromal cells or bone tissue conditioned medium. We showed that purified CXCL12, bone stromal cells, and bone tissue conditioned medium all stimulated chemomigration of PC-3 cells. Pretreatment with anti-CXCR4 antibody inhibited PC-3 cell migration towards stromal cells in a dose dependent fashion (Figure 6A). Pre-treatment of PC-3 cells with anti-CXCR4 antibody similarly inhibited bone conditioned media-induced chemomigration (Figure 6B). These data indicated that cancer cell migration in bone co-cultures is dependent on CXCL12/CXCR4 signaling.

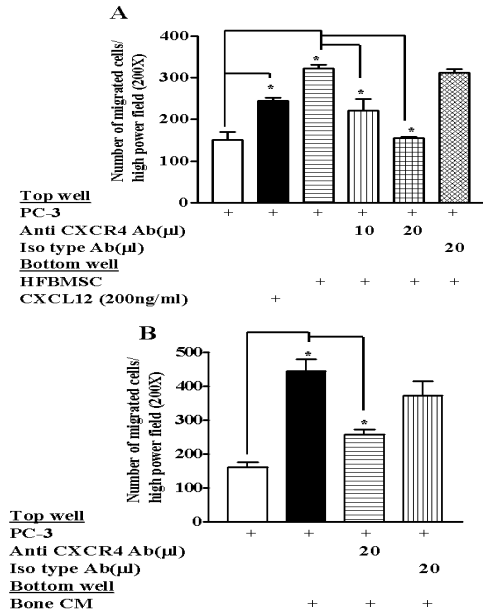

Fig 6. Migration of PC-3 cells induced by bone is CXCR4-dependent. (A) PC-3 cell migration was assessed in a transwell co-culture assay with human fetal bone stromal cells. PC-3 cells were seeded in upper chamber and human fetal bone stromal cells were seeded in bottom well. After 16 hours of incubation, the inserts were stained and number of migrated PC3 cells was scored. (B) PC-3 cell migration was assessed in a similar fashion using conditioned medium from human fetal bone tissue as a chemo attractant. Experiments were performed in triplicate, and the data were analyzed using ANOVA. The tukey post-test was performed for pair-wise comparisons, and * represents p values $\prec 0.05$.

CXCL12-mediated PC-3 cell chemoinvasion is sensitive to PI3-Kinase and MEK kinase inhibitors. The PI3 kinase and MAP kinase pathways have been shown to be activated by CXCL12/CXCR4 interaction (2, 10, 11). Pretreatment of PC-3 cells with either LY294002 (PI3 kinase inhibitor) or U0126 (MEK inhibitor) abrogated CXCL12-induced MMP-9 gene expression (Annual Report 04). One of the biological activities of cancer cell-derived MMPs are to facilitate invasion of the extracellular matrix (12, 13). Using matrigel coated cell culture inserts, we showed that pharmacological inhibition of either the PI3 kinase or MAP kinase pathway abolished CXCL12-induced chemoinvasion of PC-3 cells (Figure 7). LY294002 appeared to be more potent that U0126 in inhibiting CXCL12-induced MMP-9 gene (Figure 6) and protein expression as well as chemoinvasion in prostate cancer cells.

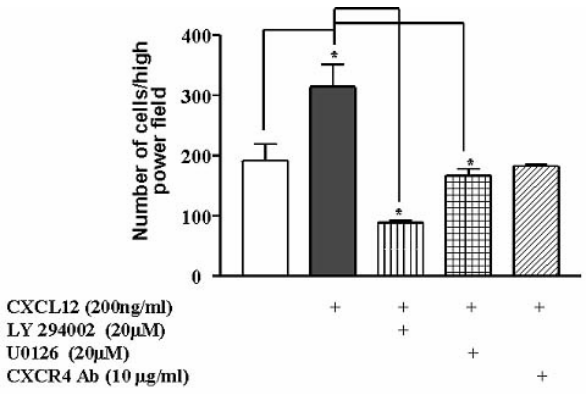

Fig 7. CXCL12-induced PC-3 cell chemoinvasion is sensitive to PI3-Kinase and MAP kinase inhibition. PC-3 cell invasion assay using matrigel-coated transwell culture inserts. CXCL12 was included in the bottom chamber as a chemoattractant. LY294002 or U0126 was included with the cancer cells in the upper chamber as indicated. The data were analyzed using ANOVA, and are presented as mean ± s.e. from triplicate experiments. Tukey post-test was used for pair-wise comparisons, and * represents p values $\prec 0.05$.

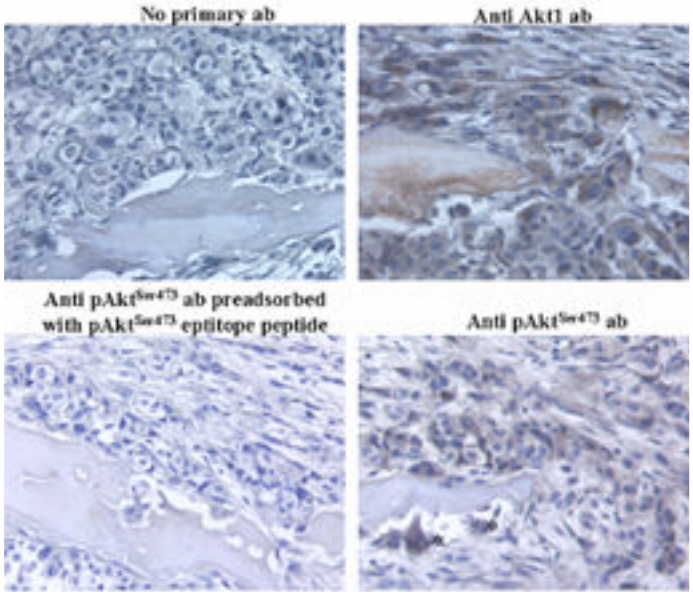

Figure 8. CXCL12 activates Akt kinase in PC-3 bone tumors. Immunohistochemical analysis of Akt1 and pSer473 Akt in SCID-human PC-3 bone tumors. No primary antibodies were used in negative control for Akt1 immunostaining and pAkt ^{Ser473} antibodies were preadsorbed with immunogenic epitope of pAkt ^{Ser473} for 30 min and entire mixture were used in the immunostaining for pAkt ^{Ser473}. * Denotes osteoclasts residing near trabecular bone.

CXCL12-induced Akt phosphorylation in PC-3 cells. Published results show that CXCL12 activates Erk 1 and 2 (2), as well as Akt (11) in different prostate cancer cell lines (11). Here, we found that CXCL12 stimulation led to activation of Akt in PC-3 cells (Annual Report 04), and this activation was sensitive to pretreatment of PC-3 cells with LY294002. These data suggested that Akt phosphorylation was mediated via the PI3 kinase pathway. Immunohistochemical analysis of SCID-human PC-3 bone tumors demonstrated specific staining for both Akt1 and pAkt^{Ser473} in both cancer cells and multinucleated osteoclasts residing near bone trabeculae (Figure 8).

Akt1 kinase activation is indispensable for CXCL12 induced MMP-9 gene expression, release, migration and invasion of PC-3 cells. Previous studies showed that the three known isoforms of Akt were expressed in prostate cancer cells (14, 15). We used a sensitive real time PCR method to quantify the relative abundance of the various Akt isoforms in our cells (Figure 9 A). We found that the Akt1 gene is the most abundant isoform expressed in all the cell lines tested. To further understand the role CXCL12-induced activation of the PI3 kinase pathway in proMMP-9 secretion, cell migration, and invasion, we employed siRNA methodology to knock down Akt1. Western blot analysis of cells transiently transfected with Akt1 siRNA showed that Akt 1 protein expression was specifically down regulated in PC-3 cells (Figure 9B). Furthermore, down regulation of the Akt1 isoform by siRNA diminished the overall levels of 473pSer-activated Akt (Figure 9C), suggesting that CXCL12 signaling involves the Akt1 isoform in PC-3 cells. We then stimulated the Akt1 siRNA-transfected cells and control-transfected cell with CXCL12 and found that MMP-9 secretion was inhibited only in the Akt1 si-RNA transfected cells (Figure 9D). Furthermore, Akt1 siRNA transfection specifically inhibited CXCL12-induced migration (Figure 9E) and invasion (Figure 9F). Together, these data showed that Akt1 signaling was unequivocally involved in CXCL12-induced MMP-9 secretion, migration, and invasion by PC-3 cells.

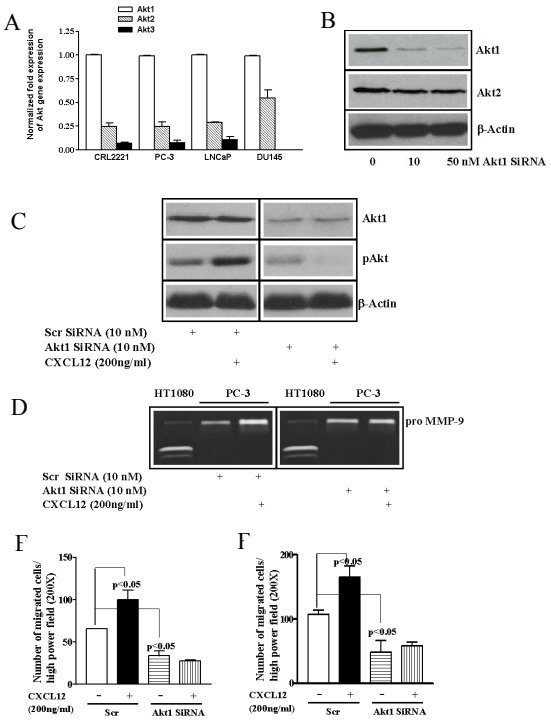

Fig 9. Akt1 oncogene is essential for CXCL12-induced MMP-9 gene expression, cell migration and invasion. (A) Real time PCR was used to assess gene expression of three isoforms of Akt in a panel of cultured cell lines. (B) Immunoblot analysis demonstrating successful knock down of Akt1 expression using a siRNA approach. (C) PC-3 cells transfected with either scrambled (scr) siRNA or Akt1 siRNA were treated with CXCL12 for 15 min. Total cellular proteins were immunoblotted with anti Akt1, anti pSer473, and anti β-actin antibodies. (D) PC-3 cells transfected with either scrambled siRNA or Akt1 siRNA were treated with CXCL12 for 16 hours. Conditioned media were subjected to gelatin zymography. (E) Migration and (F) invasion of PC-3 cells transfected with either scrambled siRNA or Akt1 siRNA and treated with 200ng/ml of CXCL12. Transfections were performed in triplicate for all the experiments, and data were analyzed using ANOVA and * represents p values ≤0.05.

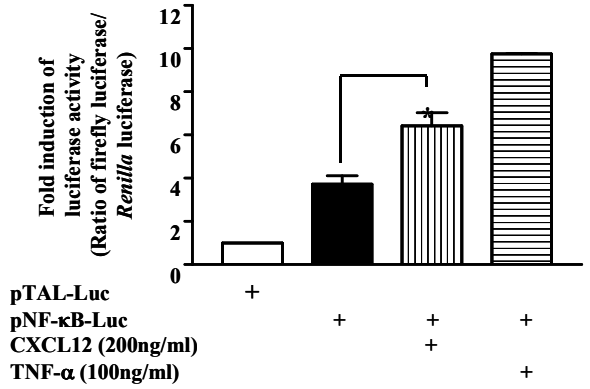

Fig 10. CXCL12 stimulates expression of the NF-κB response element. PC-3 cells were transfected with empty luciferase vector, pTAL-Luc vector, or NF-κB-Luc vector. Transfected cells were treated with either CXCL12 or TNF-α, and normalized luciferase activities are shown. TNF-α treatment used as a positive control, which has shown to activate NF-κB transcription factor. The transfections were performed in triplicate, and the data were analyzed using the student t-test. Data are expressed as mean ± s.e. and * represents p values ≤0.05

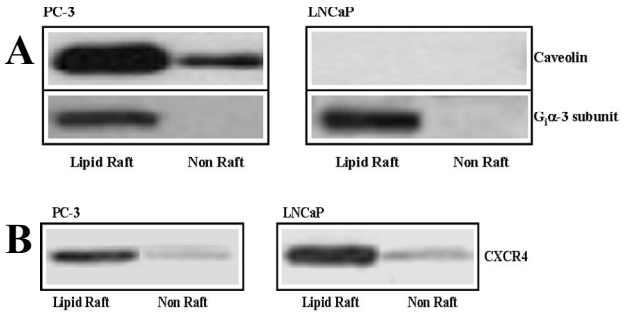

Fig 11. CXCR4 localized to lipid rafts. (A) Lipid raft fractions were isolated from prostate cancer cells using a successive detergent solubilization technique. The raft and non-raft fractions were immunoblotted with anti caveolin and anti $G_i\alpha$-3 subunit antibodies to confirm successful isolation of rafts. (B) Immunoblot showing localization of CXCR4 to lipid raft fractions.

CXCL12/CXCR4 interaction leads to activation of the NF-κB response element. Recent studies showed that activated Akt transduces signals via the cellular activation of NF-κB transcription factor (16, 17). Among the secreted MMPs, only MMP-9 has an NF-κB transcription factor binding element in the promoter sequence (18), and we previously showed that PC-3 cells are capable of responding to external stimuli by activating NF-κB transcription factor activity (19). Here we evaluated whether CXCL12/CXCR4 signaling can act via NF-κB. We transfected PC-3 cells with an NF-κB response element fused to a luciferase reporter construct. CXCL12 treatment of the transfected cells led to luciferase expression demonstrating activation of the NF-κB response element (Figure 10). These data suggest that CXCL12/CXCR4-mediated PI3K/Akt activation leads to expression of NF-κB responsive genes.

CXCR4 protein was localized to lipid rafts in PC cells: Lipid rafts are cell membrane microdomains that modulate oncogenic signal transduction by maintaining steady state levels of growth factor receptors(20, 21). To understand the role of CXCR4 signaling events during CXCL12-induced migration of cancer cells, we isolated rafts from PC3 cells using a sequential detergent solubilization technique(22). As expected, lipid raft marker $G_i\alpha$-3 localized to the triton-insoluble

portion of the plasma membrane in PC-3 cells and LNCaP cells, and caveolin localized to the triton-

Table 1: Protease gene expression changes in PC-3 cells over-expressing CXCR4. The genes shown in the table are over-expressed (fold increased) or inhibited (fold decreased) in PC-3CXCR4-2.3 cells compared to PC-3Neo cells.

Name of the gene	Fold increased	Fold decreased
Proteases:		
MMP10	34.3	
MMP13	10.6	
MMP1	4.9	
MMP9	4.6	
MMP24	2	
Cathepsin S	22.6	
Cathepsin D	4.3	
Cathepsin U	2.3	
Caspase 10	24.3	
Proteosome subunit b type 9	7.0	
Proteosome subunit b type 8 (LMP7)	3.0	
Serine Protease 16 (PRSS16)	2.1	
SUMO-1-specific protease	2	
Complement C1r-like proteinase precursor	2	
ADAMTS		0.2
ADAM12 variant		0.2
Protease inhibitor:		
TIMP1	2.1	
Antileuoproteinase inhibitor (SLPI)	3.5	
Serine protease inhibitor, Kunitz type 1 (SPINT1)	2.5	
Protease inhibitor 3, skin derived (SKLAP)	2.3	
Serine (or cysteine) proteinase inhibitor, Clade B member 2	6.5	
Serine (or cysteine) proteinase inhibitor, Clade B member 1	2.5	
Serine (or cysteine) proteinase inhibitor, Clade B member 3	2	
TIMP4		0.5

insoluble fraction of the plasma membrane in PC-3 cells (Figure 11A), confirming the successful isolation of rafts. LNCaP cells are known not to express caveolin(23). Western blot analyses of lipid raft and non-raft fractions of PC-3 and LNCaP cells showed that CXCR4 was enriched in the lipid raft fraction (Figure 11B).

CXCR4 over-expression induces protease and chemokine gene expression in PC-3 cells: CXCR4 protein was over-expressed in PC-3 cells by plasmid tranfection, and stable clones were selected and analyzed for CXCR4 over-expression. PC-3CXCR4-2.3 clone has highest CXCR4 expression among all the clones tested. For transfection control, PC-3 cells were transfected with empty vector containing neomycin resistance gene, and a clone

Table 2: Chemokine and chemokine receptor gene expression changes in PC-3 cells over-expressing CXCR4. The genes shown in the table are over-expressed (fold increased) or inhibited (fold decreased) in PC-3CXCR4-2.3 cells compared to PC-3Neo cells.

Name of the gene	Fold increased	Fold decreased
Chemokine Receptors:		
CXCR4	111.4	
GPR87	2.3	
Chemokines:		
CXCL5	68.6	
CXCL11	29.9	
CXCL10	11.3	
CXCL6	4.0	
CC20	26.0	
Gro2	27.9	
Gro3	22.6	
Gro1	7.0	

designated PC-3Neo were isolated. Both, PC-3 Neo and PC-3CXCR4-2.3 cells were cultured in 0.4m inserts, which were previously coated with matrigel. The total RNA was isolated from the cells, and digested with DNAase I to degrade residual DNA contamination. The resulting RNA was send to core-facility for gene expression analysis with Affymatrix human genome chip (HGU133B). The genes which are up or down regulated were identified between PC-3Neo and PC-3CXCR4-2.3 cells, and the data were presented in Table 1 and Table 2.

KEY RESEARCH ACCOMPLISHMENTS

- *In vitro* and *in vivo* prostate cancer cells express CXCR4 receptor and bone associated cells express the CXCR4 ligand CXCL12.
- CXCL12 and CXCR4 interaction in prostate cancer cells induce MMP-9 gene expression and promoter activation. In PC-3 cells CXCL12 can induce the expression and secretion of pro MMP-9
- CXCL12 can activate the PI3 kinase/Akt pathway in PC-3 cells.
  Relative role of PI3K/MAPK pathway studies with well accepted pharmacological inhibitors suggest that CXCL12 induced MMP-9 gene expression in PC-3 cells are sensitive to both pathways and assessing relative sensitivity of these two inhibitors suggest that PI3K/Akt pathway is more potent in inducing MMP-9 gene expression.
- Bone tissue associated CXCL12 stimulates CXCR4 dependent PC-3 cell chemomigration.
- CXCL12 mediated chemoinvasion of PC-3 cells is sensitive to both PI3 kinase and MAP kinase inhibitors.
- SCID-human PC-3 bone tumors express Akt protein and activated Akt protein. Tumor cells and osteoclasts show immunoreactivity for both Akt and phosphorylated Akt protein.
- Akt 1 phosphorylation is indispensable for CXCL12 induced MMP-9 gene expression, secretion, migration and invasion of PC-3 cells.
- CXCL12/CXCR4 interactions activates NF-κB response element in PC-3 cells.
- Gene expression profiles that several members of protease family and chemokine family were up-regulated in CXCR4 over-expressing PC-3 cells compared to corresponding Neo cells.
- CXCR4 was localized to lipid rafts fractions of PC cells.

REPORTABLE OUTCOMES

~~Podium Presentations~~:

Chinni SR.

CXCR4 and MMP-9 in prostate cancer bone metastasis. The IVth International conference on cancer induced bone diseases. San Antonio, TX. December 2003.

Role of CXCR4 and MMP-9 in prostate cancer bone metastasis. Plenary lecture. Vth Annual Michigan Prostate Research Colloquium on basic and clinical advances of prostate cancer. April 23, 2005.

~~Abstract~~:

Chinni, SR, Sivalogan S, Dong Z, Trindade Filho JC, Bonfil RD and Cher, ML. CXCL12 chemokine-CXCR4 receptor interaction in prostate cancer induces akt signaling, mmp-9 expression, motility and invasion. American Urological Association annual meeting, San Antanio, TX. May 21-26, 2005.

Chinni, SR, Sivalogan S, Dong Z, Trindade Filho JC, Bonfil RD and Cher, ML. The bone microenvironment induces CXCL12/CXCR4 signaling, Akt activation, and MMP-9 expression in prostate cancer cells. IV symposium on skeletal complications of malignancy, NIH, Bethesda, MD. April 28-30, 2005

Chinni, SR, Sivalogan S, Dong Z, Trindade Filho JC, Bonfil RD and Cher, ML. CXCL12 and CXCR4 interaction in prostate cancer induces akt signaling, mmp-9 expression, motility and invasion 96th Annual Meeting of American Association for Cancer Research. Anaheim, CA. CD-ROM, 2005.

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Chinni, SR, Sivalogan S, Dong Z, Trindade Filho JC, Bonfil RD and Cher, ML. CXCL12/CXCR4 signaling induces Akt activation and MMP-9 expression in prostate cancer cells. Eleventh Annual Prostate Cancer Foundation Scientific Retreat, Lake Tahoe, NV. October 21-24, 2004.

Chinni, SR, Sivalogan S, Dong Z, Trindade Filho JC, Bhagat S; Bonfil RD and Cher, ML. Akt activation and MMP-9 secretion in prostate cancer bone interaction. *Proceedings of 95th Annual Conference*, American Association for Cancer Research. CD-ROM, 2004.

Chinni SR, Dong Z, Sivalogan, S, Trindade Filho JC, Bhagat S, Bonfil R and Cher ML. CXCR4 and MMP-9 in prostate cancer bone metastasis. The IVth international conference on cancer-induced bone diseases. December 2003; p25.

Chinni SR, Deng X, Dong Z, Bhagat S, Bonfil RD, Cher ML. The Role of SDF-1α/CXCR4/MMP-9 in Prostate Cancer Bone Metastasis. J Urol 169:354A, 2003.

~~Publication:~~

Chinni SR, Sivalogan S, Dong Z, Trindade Filho, JC, Deng X, Bonfil RD and Cher ML. CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12. *The Prostate*. 66(1):32-48, 2006.

CONCLUSIONS

These results suggest that chemokine, CXCL12 and its cognate receptor CXCR4 are expressed in the cellular phenotypes of prostate cancer bone metastasis. Bone associated CXCL12 could act as a chemoattractant for circulating Prostate Cancer cells. Biological activities of prostate cancer cell associated CXCL12/CXCR4 interactions include expression and secretion of MMP-9. CXCL12 induced cell signaling pathways mediate chemoinvasion of cancer cells, and activated Akt 1 kinase is indispensable for CXCL12 mediated cellular migration and invasion of prostate cancer cells. CXCR4 was localized to lipid raft fractions in plasma membrane of PC cells. Studies with CXCR4 over-expression in PC-3 cells reveal that several proteases including MMP-9, and chemokine ligands and receptors are down-stream targets in PC cells.

This data suggests that chemoattractive mechanisms may involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent release of MMPs into the local environment. These findings may provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover.

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APPENDICES

Appendix 1

CXCL12 CHEMOKINE-CXCR4 RECEPTOR INTERACTION IN PROSTATE CANCER INDUCES AKT SIGNALING, MMP-9 EXPRESSION, MOTILITY AND INVASION

Sreenivasa R. Chinni, Sivasakthy Sivalogan, Zhong Dong, J. Carlos Trindade Filho, R. Daniel Bonfil and Michael L. Cher*

Departments of Urology and Pathology
Wayne State University

Introduction and Objective(s): Hematopoietic cells home to bone by means of chemo-attraction to marrow chemokines, and chemokine-receptor binding leads to the expression/activation of adhesion molecules and proteases. Recent evidence suggests that a similar mechanism may be active in cancer metastasis. Previously, we showed that inhibition of matrix metalloprotease (MMP) activity diminished bone matrix turnover and proliferation of prostate cancer (PC) cells in bone. Using specific tissue-based enzymatic activity assays, we also demonstrated an increase in net MMP-9 activity, but not MMP-2 or MT1-MMP activity, in bone tissues colonized by PC cells *in vivo*. Herein, we hypothesized that CXCL12 chemokine-CXCR4 receptor interactions facilitate PC bone metastasis by activating intracellular signaling pathways leading to the expression and release of MMP-9.
Methods: We used a variety of methods including RT-PCR, immunohistochemistry, ELISA, gelatin zymography, cellular motility and invasion, and subcellular fractionation of prostate cancer cells applied to *in vivo* and *in vitro* models.
Results: Here we showed that (a) CXCR4 was expressed on the surface of PC cells and in experimental *SCID*-human bone tumors, and CXCL12 was expressed by stromal cells in bone and lung tissue; (b) CXCL12 induced MMP-9 gene expression and secretion in PC cells; (c) bone stromal cells and bone tissue conditioned media induced CXCR4-dependent migration of PC-3 cells; (d) pharmacological inhibition of PI3 kinase and MAP kinase pathways abrogated CXCL12-induced MMP-9 gene expression, secretion, and invasion of PC-3 cells; (e) CXCL12 induced Akt phosphorylation, and Akt1 siRNA transfections abrogated CXCL12-induced Akt phosphorylation, proMMP-9 secretion, migration and invasion of PC-3 cells; (f) CXCR4 was localized to lipid rafts in prostate cancer cells; and (g) CXCL12 induced HER2 phosphorylation in PC-3 cells.
Conclusions: These data suggest that chemoattractive mechanisms may be involved in migration of PC bone metastasis. Chemokine-receptor interactions lead to activation of signaling pathways ultimately resulting in release of MMP-9 into the local environment and PC cell motility and invasion. These findings provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover.

Funding: DoD

CXCL12 and CXCR4 INTERACTION IN PROSTATE CANCER INDUCES AKT SIGNALING, MMP-9 EXPRESSION, MOTILITY AND INVASION

Sreenivasa R. Chinni, Sivasakthy Sivalogan, Zhong Dong, J. Carlos Trindade Filho, R. Daniel Bonfil and Michael L. Cher*

Departments of Urology and Pathology
Wayne State University

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Conclusions: These data suggest that chemoattractive mechanisms may be involved in migration of PC bone metastasis. Chemokine-receptor interactions lead to activation of signaling pathways ultimately resulting in release of MMP-9 into the local environment and PC cell motility and invasion. These findings provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover.

CXCL12/CXCR4 SIGNALING INDUCES AKT ACTIVATION AND MMP-9 EXPRESSION IN PROSTATE CANCER CELLS

Sreenivasa R. Chinni, Sivasakthy Sivalogan, Zhong Dong, J. Carlos Trindade Filho, R. Daniel Bonfil and Michael L. Cher.

Departments of Urology and Pathology
Wayne State University and The Barbara Ann Karmanos Cancer Institute

Chemokines and chemokine receptor interactions facilitate the physiological migration of cells. Hematopoietic cells home to bone by means of chemo-attraction to marrow chemokines. Interaction of chemokines with their receptors leads to the expression/activation of adhesion molecules and proteases. Recent evidence suggests that a similar mechanism may be active in cancer metastasis: expression of the chemokine CXCL12 and its receptor CXCR4 has been documented in several epithelial tumors. We previously showed that broad-spectrum inhibition of matrix metalloprotease (MMP) activity diminishes bone matrix turnover and proliferation of prostate cancer (PC) cells in bone. Using specific tissue-based enzymatic activity assays, we recently demonstrated an increase in net MMP-9 activity, but not MMP-2 or MT1-MMP activity, in bone tissues colonized by cancer cells *in vivo*. The MMP-9 protein immunolocalized to prostate cancer cells and osteoclasts. Herein, we hypothesize that CXCL12 and CXCR4 interactions facilitates the metastasis of prostate cancer cells by activating intracellular signaling pathways leading to the expression and release of MMP-9. Using a variety of methods including RT-PCR, ELISA, gelatin zymography, cellular motility and invasion and subcellular fractionation of prostate cancer cells, we showed that (a) CXCR4 was expressed on prostate cancer cells and experimental *scid*-human PC-3 bone tumors and CXCL12 was expressed on stromal cells of bone and lung and to a lesser extent in PC-3 cells; (b) CXCL12 induced MMP-9 gene expression and secretion in prostate cancer cells; (c) bone stromal cells and bone tissue conditioned media induced the migration of PC-3 cells in a CXCR4 dependent manner; (d) pharmacological inhibition of PI3 kinase and MAP kinase pathways abrogated the CXCL12 induced MMP-9 gene expression and release, and invasion of PC-3 cells; (e) CXCL12 induced Akt1 phosphorylation and Akt1 siRNA transfections abrogated the CXCL12 induced Akt phosphorylation, proMMP-9 secretion, migration and invasion of PC-3 cells; (f) CXCR4 was localized to lipid rafts in prostate cancer cells and CXCL12 induced HER2 phosphorylation in PC-3 cells; (g) kinetics of CXCL12 induced HER2 and Akt phosphorylation showed that CXCL12 induced HER2 phosphorylation preceded Akt phosphorylation in PC-3 cells. This data suggests that chemoattractive mechanisms may involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent release of MMPs into the local environment. These findings may provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover.

Akt activation and MMP-9 secretion in prostate cancer bone interaction

Chinni, SR; Sivalogan S; Dong, Z; Trindade Filho, JC; Bhagat, S; Bonfil RD and Cher, ML

Departments of Urology and Pathology
Wayne State University and The Barbara Ann Karmanos Cancer Institute

We showed previously that broad-spectrum inhibition of matrix metalloprotease (MMP) activity diminishes bone matrix turnover and proliferation of prostate cancer (PC) cells in bone (JNCI 94:17, 2002). Using specific tissue-based enzymatic activity assays, we recently demonstrated an increase in net MMP-9 activity, but not MMP-2 or MT1-MMP activity, in bone tissues colonized by cancer cells *in vivo*. The MMP-9 protein immunolocalized to prostate cancer cells and osteoclasts (Proc AACR 44:3269A, 2003). Herein, we sought to characterize intracellular signaling pathways that lead to the secretion of MMP-9 by cancer cells exposed to the bone microenvironment. The PI3K/Akt signaling pathway is known to be activated in variety of cancer cells following interaction with extracellular matrix components and/or autocrine/paracrine growth factors; thus, we analyzed this pathway during co-culture of PC cells with human bone marrow stromal cells or intact bone tissue. Using a variety of methods including confocal analysis, western blot, and gelatin zymography, we showed that (a) secretion of pro-MMP-9 by PC-3 cells is induced by co-culture with bone stromal cells; (b) the Akt pathway is selectively activated in PC-3 cells during co-cultures; (c) secretion of proMMP-9 by PC-3 cells is sensitive to the PI3K inhibitor, LY294002; (d) proteolytic activation of latent pro-MMP-9 to active MMP-9 occurs when PC-3 cells are cultured together with intact bone tissue; and (e) the activation of MMP-9 is sensitive to PI3K inhibition by LY294002. Western blot analysis of experimental *in vivo* PC bone tumor tissue showed activation of Akt and its downstream molecules. These data suggest that activation of tumor cell-associated Akt kinase and downstream molecules in the Akt pathway play a key role in bone stromal-tumor interactions leading to the secretion of MMP-9 by tumor cells. Neutralization strategies involving dominant negative Akt constructs and isoform-specific Akt siRNA are under progress to delineate the mechanisms underlying Akt signaling and MMP-9 expression and activation.

CXCR4 and MMP-9 in prostate cancer bone metastasis

Chinni, SR; Dong, Z; Sivalogan S; Trindade Filho, JC; Bhagat, S; Bonfil RD and Cher, ML

Wayne State University

Mechanims underlying organ-specific metastasis to bone likely include both chemoattractive homing phenomena and selective proliferative advantages of cancer cells after their arrival in the marrow. Recent evidence implicates the chemokine SDF-1α and its receptor CXCR4 in organ-specific metastasis of breast cancer. Previously, we demonstrated that circulating prostate cancer (PC) cells selectively colonize human fetal bones implanted in SCID mice and that matrix metalloproteinases, particularly MMP-9, play a role in the proliferation of PC cells in bone and subsequent bone matrix remodeling. Herein, we injected PC3 cells into fetal human bone implants, and harvested the tissues at 1, 3 days for their microscopic location and 14 days for MMP-9 activity measurements. Using several methods, we determined the expression of CXCR4 in prostate cancer bone tumor cells and cultured PC cells, and the effect of both exogenous SDF-1α and co-cultures of prostate cancer cells and bone stromal cells on the secretion of proMMP-9. PC cells injected directly into implanted bones at early time periods migrated toward endosteal surfaces of trabeculae suggesting a chemoattractive-migratory mechanism. *In vitro* treatment of PC-3 cells with SDF-1α and co-cultures of cancer cells and stromal cells showed a dose-dependent effect on the secretion of proMMP-9. PI3 Kinase inhibitors abrogated MMP-9 secretion. These data suggest that chemoattractive mechanisms stimulate the migration of cancer cells towards mineralized trabeculae, and that PI3 kinase signaling induced by SDF-1α and CXCR4 releases MMPs into local environment.

Appendix 2

The Prostate 66:32–48 (2006)

CXCL12/CXCR4 Signaling Activates Akt-1 and MMP-9 Expression in Prostate Cancer Cells: The Role of Bone Microenvironment-Associated CXCL12

Sreenivasa R. Chinni,[1,2]* Sivasakthy Sivalogan,[1]
Zhong Dong,[1] J. Carlos Trindade Filho,[1] Xiyun Deng,[1]
R. Daniel Bonfil,[1,2] and Michael L. Cher[1,2]

[1]*Department of Urology, Wayne State University School of Medicine and The Barbara Ann Karmanos Cancer Institute, Detroit, Michigan*

[2]*Department of Pathology, Wayne State University School of Medicine and The Barbara Ann Karmanos Cancer Institute, Detroit, Michigan*

BACKGROUND. Hematopoietic cells home to bone by means of chemo-attraction to marrow chemokines, and interaction of chemokines with their receptors leads to the expression/activation of adhesion molecules and proteases. Recent evidence suggests that similar mechanisms may be active in cancer metastasis. Previously, we showed that metalloproteases (MMPs), and in particular MMP-9, play a role in prostate cancer (PC) expansion in bone.
METHODS. We used a variety of methods including RT-PCR, immunohistochemistry, ELISA, gelatin zymography, cellular motility and invasion, and subcellular fractionation of PC cells applied to in vivo and in vitro models.
RESULTS. Here we showed that (a) CXCL12/CXCR4 axis is expressed in PC bone metastasis; (b) exogenous CXCL12 induced MMP-9 expression by PC cells; (c) bone stromal cells and bone tissue conditioned media induced the migration of PC cells in a CXCR4-dependent manner; (d) pharmacological inhibition of PI3 kinase and MAP kinase pathways abrogated CXCL12-induced MMP-9 expression and invasion of PC cells; (e) exogenous CXCL12 induced Akt1 phosphorylation is indispensable for proMMP-9 secretion, migration, and invasion of PC cells; (f) CXCR4 was localized to lipid rafts in PC cells and initiated Akt phosphorylation.
CONCLUSIONS. These data suggest that chemoattractive mechanisms involve migration of cancer cells towards bone tissue, and that cell signaling induced by binding of the chemokine to its receptor leads to the activation of multiple signaling pathways and subsequent secretion of MMP-9 into the local environment. These findings provide a link between chemoattractive mechanisms, growth of tumor cells in bone, and tumor-enhanced bone matrix turnover. *Prostate 66: 32–48, 2006.* © 2005 Wiley-Liss, Inc.

KEY WORDS: **CXCL12; CXCR4; MMP-9; Akt; PC and bone metastasis**

INTRODUCTION

Prostate cancer (PC) is the second leading cause of death in men [1], and metastatic growth at distant organs is the primary cause of morbidity and mortality. Bone is the preferred organ site for metastasis of PC [2]. The basis for organ-specific metastasis can be explained in general by Paget's "seed and soil" hypothesis [3] wherein particular metastatic sites provide favorable growth conditions for specific types of cancers. Biologic

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*Correspondence to: Sreenivasa R. Chinni, PhD, Departments of Urology and Pathology, Wayne State University School of Medicine, 9105 Scott Hall, 540 E. Canfield Avenue, Detroit, MI 48201.
E-mail: schinni@med.wayne.edu

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explanations for this broad hypothesis include [1] enhanced tumor cell proliferation in specific organ microenvironments (reviewed in Ref. [4]); [2] enhanced adhesion of circulating cancer cells to endothelia at specific sites [5]; and [3] chemoattraction of circulating cancer cells toward chemokines released by specific sites [6].

Chemokines are a 8–10 kDa peptides. There are approximately 45 family members [7] organized into four classes (CXC, CC, C, and CX3C) based on their N-terminal cysteine motif. Chemokine receptors are G-protein coupled receptors (GPCR) with seven-trans-membrane domains. The chemokine CXCL12, formerly known as SDF-1α, and its receptor CXCR4 have been studied extensively with regard to hematopoietic and immune cell functions. CXCL12 is the only known ligand for CXCR4. CXCL12 and CXCR4 were found to be involved in the development of several subsets of immune cells and their migration processes [7]. A recent report suggested that CXCL12 and CXCR4 are involved in the metastasis of breast cancer to lymph nodes [6]. Subsequent studies showed that CXCL12/CXCR4 interactions are broadly involved in variety of cancers (reviewed in Ref. [8]).

Cell surface expression of CXCR4 is regulated at several levels including intracellular signaling events controlling *CXCR4* gene transcription [9–13] and receptor internalization following ligand binding [14]. In tumor cells, CXCR4 expression is trasncriptionally regulated by the RET oncogene [9], the von Hippel-Lindau tumor suppressor gene [10], hypoxia-inducible factor (HIF-1α) [12], and NF-κB transcription factor [11]. Interestingly, mammary fat pad and bone micro-environments have been shown to induce *CXCR4* gene expression in breast cancer cells [11,13]. CXCL12-induced cell migration requires the activation of CXCR4-dependent $G_{i2}\alpha$ proteins. Downstream intra-cellular signaling pathways activated via CXCR4-dependent $G_{i2}\alpha$ proteins include the PI3 kinase, PKC, and MAP kinase pathways, and the contribution of activated pathways to CXCL12-induced cell migration appears to be cell-type specific [15]. CXCL12-mediated Akt activation confers multiple activities in different cell types: trans-endothelial migration of breast cancer cells [16] and T lymphocytes [17], migration of small cell carcinoma cells [18], proliferation of brain [19], and ovarian [20] cancer cells, and survival of brain cancer cells [21]. Several types of proteases are expressed upon CXC12/CXCR4 activation, and protease activity is thought to be crucial for migration of variety of cells including leukocytes, osteoclast precursor cells, and epithelial cancer cells [22–24].

PC cell migration is one of many events in the development of clinically evident bone metastases. Upon arrival in bone, cells must adhere, invade, proliferate, and stimulate bone remodeling and angio-genesis. Using animal models of bone metastasis, we and others showed that tumor growth in bone and tumor-associated bone remodeling can be inhibited by systemic administration of small molecule broad-spectrum MMP inhibitors [25,26]. Using specific enzy-matic activity assays, we recently demonstrated an increase in net MMP-9 activity, but not MMP-2 or MT1-MMP activity, in bone tissues colonized by PC cells. The MMP-9 protein was localized to cancer cells and osteoclasts [27]. Herein, we hypothesized that CXCL12/CXCR4-mediated chemoattraction of tumor cells to bone could be linked to MMP-mediated intra-osseous tumor expansion and bone remodeling. To this end, we sought to determine if CXCL12/CXCR4-mediated signaling events could be linked to MMP-9 expression and release by tumor cells. We found that the bone environment induced CXCR4-dependent migration. Furthermore, CXCL12/CXCR4 signaling induced *MMP9* gene expression and secretion and was functionally active in migration and invasion of cancer cells. MMP-9 expression and tumor cell migra-tion was found to be dependent on Akt1 kinase activation.

MATERIALS AND METHODS

Cell Culture

PC-3, LNCaP, DU145, and CRL2221 cells were obtained from American Type Culture Collection (Manassas, VA). PC-3 and LNCaP cells were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% FBS and 1% Penicillin and Streptomycin. DU145 cells were cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin and Streptomycin. CRL2221 were cultured in kerati-nocyte-SFM medium supplemented with 10% FBS, 1% Penicillin and Streptomycin, 0.2 μg/L Epidermal Growth Factor, 30 mg/L bovine pituitary extract.

Gelatin Zymography

Conditioned media from PC-3 cells treated with CXCL12 (Peprotech, Rocky Hill, NJ) and/or pharma-cological inhibitors, LY294002 (Sigma-Aldrich, St. Louis, MO) or U0126 (Calbiochem, San Diego, CA) were collected, and equal volumes and/or equal amounts of protein from particulate-free conditioned medium were mixed with sample buffer (75 mM Tris-HCl, pH 6.8, 0.1% SDS, 10% glycerol, and bromophenol blue) and analyzed on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin. After electrophoresis, the gel was incubated in renaturing buffer (2.5% Triton X-100) at room temperature for 30 min, washed twice with distilled water (20 min each time), and then incubated

with developing buffer (50 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 200 mM NaCl, and 0.02% Brij-35) for 30 min. Finally, the gel was developed in fresh developing buffer at 37°C overnight, stained in 0.5% Coommassie Blue solution for 2 hr, and destained in destaining buffer (5% acetic acid, 10% methanol) until bands of gelatinolytic activity were visualized. Recombinant proMMP-9 or conditioned medium from human fibrosarcoma HT-1080 cells were used as a positive controls. A representative zymogram from three independent experiments was shown.

Luciferase Reporter Transfections

PC-3 cells were transfected with either a pGL3-MMP-9 plasmid containing 620 bp of the MMP-9 promoter cloned into the pGL3 basic plasmid [28] or a NF-κB-Luc plasmid DNA containing four tandem copies of the NF-κB response element present proximal to the TATA-like promoter (Clontech, Palo Alto, CA). For MMP-9 promoter studies, the *Renilla* Luciferase vector pRL-CMV (Promega, Madison, WI) was co-transfected to serve as an internal control for normalizing transfection efficiency. Similarly, the pTAL (Clontech, Palo Alto, CA) vector was used as an internal control for the NF-κB response element reporter studies. 5×10^5 cells were transfected with plasmid DNA using lipofectamine 2000 (Invitrogen). Twenty-six hours after transfection, cells were serum-starved for 4 hr and then treated with ligands or pharmacological reagents overnight. Fourty eight hours after transfection, cell lysates were prepared in lysis buffer and 10 μl of lysate was used with the dual luciferase reporter assay system (Promega). Luciferase activity was integrated over a 10 second time period in a TD-20e luminometer (Turner Designs, Sunnyvale, CA). Cell lysates were assayed for luciferase and *Renilla* luciferase activities successively in the same vial as per the manufacturer's recommendations. The luciferase activity in each sample was normalized to *Renilla* luciferase activity before calculating fold activation. For calculation of fold activation of the MMP-9 promoter and NF-κB response element activities, the luciferase activity from the vector controls of pGL3 basic and pTAL were arbitrarily set at 1. The data presented are mean ± SE of three independent transfection experiments. *P* values were calculated using the Student *t*-test.

Real-Time PCR

Following treatment with either ligand or combination of ligand and pharmacological inhibitors, 4×10^5 cells were seeded in 6 well plates, and total RNA was isolated using Trizol reagent (Invitrogen). For RT-PCR studies, first-strand cDNA was synthesized from 2 μg of total RNA with an oligo (dT) primer and Super Script II reverse transcriptase (Invitrogen). Forward and reverse primers were based on published reports (human MMP-9 [29]: 5′-TGG GCT ACG TGA CCT ATG ACA T-3′ and 5′-GCC CAG CCC ACC TCC ACT CCT C-3′; human CXCL12 [30]: 5′-TGC CAG AGC CAA CGT CAA G-3′ and 5′-CAG CCG GGC TAC AAT CTG AA-3′; human Akt1 [31]: 5′-ATG AGC GAC GTG GCT ATT GTG AAG-3′ and 5′-GAG GCC GTC AGC CAC AGT CTG GAT G-3′; human Akt2 [31]: 5′-ATG AAT GAG GTG TCT GTC ATC AAA GAA GGC-3′ and 5′-TGC TTG AGG CTG TTG GCG ACC-3′; human Akt3 [31]: 5′-ATG AGC GAT GTT ACC ATT GT-3′ and 5′-CAG TCT GTC TGC TAC AGC CTG GAT A-3′; human GAPDH [29]: 5′-AAG GTC ATC CCT GAG CTG AA-3′ and 5′-TGA CAA AGT GGT CGT TGA GG-3′). For human CXCR4, following primers were designed using the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): 5′-GGC CCT CAA GAC CAC AGT CA-3′ for forward primer, and 5′-TTA GCT GGA GTG AAA ACT TGA AG-3′ for reverse primer. The real-time PCR analysis was performed with SYBER green PCR core reagents (Stratagene, La Jolla, CA) in a Stratagene M × 4000 cycler, and the data analysis was performed using M × 4000 v3.01 software. All primer sets were tested in real-time PCR and found to produce no detectable peaks in dissociation curves due to primer–dimer amplifications. The amplicon size ranges from 60 nucleotides to 325 nucleotides. Relative message levels were calculated with a comparative Ct (Threshold cycle method) method [32]. Briefly, message levels were normalized to endogenous GAPDH message levels. In treated samples, relative quantitation was performed by the comparative Ct method [32] using the formula 2^{-Ct}, where Ct = [Ct test gene (treated sample) − Ct GAPDH (treated sample)] − [Ct test gene (control sample) − Ct GAPDH (control sample)]. For each sample, real-time PCR was performed in triplicate samples, Ct represents the mean Ct value of each sample, and GAPDH is the endogenous control used to normalize the quantification of a test gene.

Fluorescence Activated Cell-Sorting Analysis (FACS)

PC cells grown in culture plates were scraped and counted on hemocytometer. 5×10^5 cells were resuspended in phosphate buffered saline supplemented with 5% fetal bovine serum and incubated with either phycoerythrin (PE) conjugated anti CXCR4 antibody (BD Pharmingen, San Diego, CA) or isotype matched IgG_{2a} (BD Pharmingen) for 15 min on ice. Antibody bound cancer cells were washed for three times and analyzed on fluorescence activated cell sorter (Becton Dickenson, San Diego, CA). CXCR4 positive cells were

enumerated using the cell quest software (Becton Dickenson).

ELISA

Human fetal femurs, obtained as previously described [25,33], were used either for bone organ culture experiments or to obtain primary cultures of bone marrow stromal cells. For ELISA assays, femurs were cut into six small fragments and placed in 24 well plates. Alternatively, bone marrow stromal cells were seeded at a density of 2×10^5/well. The following day, bone fragments or stromal cells were serum-starved for 4 hr and then exposed to 0.5 ml serum-free RPMI 1640 supplemented with 1% Penicillin and Streptomycin. Media were collected at 24, 48, and 72-hr intervals. CXCL12 levels were quantified in triplicate samples using a quantikine kit for human SDF-1α (CXCL12) according to the manufacturer's recommendations (R & D Systems, Inc., Minneapolis, MN) and expressed as picograms/mg of total protein.

Invasion and Migration Assays

Cancer cells were serum-starved for 4 hr. $1.5–2.0 \times 10^5$ cells were seeded on to inserts in the upper chamber of trans well culture plates (Becton Dickenson). To neutralize CXCL12-induced invasion and migration of cancer cells, the cells were pretreated with neutralizing anti-CXCR4 antibody (12G5), which has shown to inhibit CXCL12-induced migration of human cells [34]. For invasion studies, the filters were pre-coated with matrigel. Cell migration and invasion were allowed to proceed for 16 and 24 hr, respectively. Later, the upper chambers were cleaned with cotton swabs to remove non-migrated/invaded cells and the inserts were stained with Diff-Quik stain set (Dade Behring Inc., Newark, DE).

Western Blot Analysis

2×10^5 cancer cells were seeded on to 100 mM plates. The following day, the cells were serum-starved for 5 hr and then exposed to either CXCL12, Epidermal Growth Factor (Invitrogen Life Technologies), and LY294002. Total cellular proteins were extracted with buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 1 mM PMSF, and 1 × Protease inhibitor cocktail (Roche, Indianapolis, IN). Protein content was quantified with a BCA protein assay (Pierce, Rockford, IL), and equal amounts of protein were resolved by 8% SDS PAGE. Immunoblot was performed with antibodies to Akt1, $pAkt^{Ser473}$ (Cell Signaling, Beverly, MA) β Actin (Sigma-Aldrich). A representative blot from three independent experiments was shown.

Akt1 siRNA Synthesis and Transfection of PC-3 Cells

The published mouse Akt1-specific small interfering RNA sequence (siRNA) [35] was used as a basis to design an analogous human siRNA for Akt1. The mouse siRNA sequence corresponded to the human sequence 5′-AAC CAG GAC CAC GAG CGC CTC-3′ taken from GenBank accession number NM_005163. This sequence has 91% identity with mouse sequence. The Silencer™ siRNA construction kit (Ambion Inc, Austin, TX) was used to design the sense and antisense oligos, and an in vitro transcription reaction was performed to generate Akt1 siRNA. PC-3 cells were transfected with 10–50 nM of Akt1 siRNA or scrambled siRNA (Ambion Inc.) using lipofectamine 2000 reagent. Western blot analysis was performed with total cellular proteins for Akt1, Akt2, and β-actin. Transfected cells were treated with CXCL12 and assessed for invasion and migration; conditioned media were analyzed by zymography. All the transfection experiments were performed in triplicate and a representative Western blot or zymogram was shown.

Immunohistochemistry of SCID-Human PC-3 Bone Tumors

1×10^6 PC-3 cells were injected into human fetal femur fragments previously implanted into SCID mice according to previously described methods [25,33]. Six days later, the bones were excised, decalcified, sectioned, and stained with anti bodies against CXCR4 (R & D Systems, Inc.), CXCL12 (eBioscience, San Diego, CA), Akt1, and $pAkt^{Ser473}$ antigens using the Vecta Stain ABC kit (Vector Laboratories, Inc., Burlington, CA).

Lipid Raft Isolation

Monolayer cultures of PC-3 and LNCaP cells were serum-starved for 6 hr and treated with CXCL12 (200 ng/ml). Previously described sequential detergent solubilization techniques [36] were modified slightly and used to fractionate plasma membrane-associated lipid rafts. The cells were washed with PBS, scraped, and resuspended in buffer A containing 25 mM 2-Morpholinoethanesulfonic acid (MES) (pH 6.5) and 150 mM sodium chloride. Equal amounts buffer B containing 25 mM MES (pH 6.5) and 150 mM Sodium Chloride, 2% Triton-X 100, 5 mM Sodium Floride (NaF), 2 mM Sodium OrthoVanadate (Na_3VO_4), 2 mM PMSF, and 1 × protease inhibitor cocktail (Roche, Indianapolis, IN) were added to lyse the cells and incubated on ice for 30 min. The cell lysates were centrifuged at 10,000*g* for 20 min. The soluble fractions were collected in an eppendorf tube and pellets were resuspended in buffer C containing 10 mM Tri-HCl (pH 7.6) 60 mM

β-Octylglucoside, 500 mM NaCl, 2.5 mM Sodium Fluoride (NaF), 2 mM Sodium OrthoVanadate (Na_3VO_4), 2 mM PMSF, and 1 × protease inhibitor cocktail (Roche) and incubated on ice for 30 min. The samples were centrifuged at 10,000*g* for 20 min and soluble fraction enriched with lipid raft were collected. Western blot analyses were performed with the lipid raft and non-raft fractions with antibodies against $G_i\alpha$-3 subunit (EMD Biosciences, San Diego, CA), caveolin (Transduction Labs, San Diego, CA), $pAkt^{Ser473}$, $pAkt^{Thr308}$, and CXCR4 (Chemicon International Inc., Temecula, CA).

Statistical Analysis

Statistical significance was determined by the Student *t*-test and the non-parametric one-way ANOVA test followed by Tukey posttest to compare all pairs of a column using GraphPad Prism software version 3.0 (GraphPad, San Diego, CA). $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Expression of CXCR4 in Cultured Prostate Cancer Cells and Experimental Bone Metastasis Tissue

RT-PCR analysis showed that PC cell lines PC-3, LNCaP, and DU145 expressed the *CXCR4* gene, whereas CXCR4 mRNA was nearly undetectable in immortalized prostate epithelial cells (CRL2221) (Fig. 1A). FACS analysis showed that a significant fraction of PC-3 and LNCaP cells had cell surface CXCR4 protein expression. In contrast, very few DU145 cells had cell surface expression (Fig. 1B). Immunostaining of SCID-human PC-3 bone tumor tissue for CXCR4 protein demonstrated presence of CXCR4 protein in cancer cells populating the bone marrow of implanted bone fragments (Fig. 1C).

Expression of CXCL12 in Bone Stromal Cells, Fetal Bone Tissue, and Cancer Cells

Transient primary cultures of stromal cells were established from human fetal bone and lung tissues. RT-PCR analysis of CXCL12 chemokine gene expression showed high levels of CXCL12 mRNA in bone stromal cells, intermediate levels in lung stromal cells, and nearly undetectable levels in PC cells (Fig. 2A). Soluble CXCL12 protein levels were measured in the conditioned medium of human fetal bone stromal cells and cultured human fetal bone tissue. Human fetal bone stromal cells and human fetal bone tissue secreted soluble CXCL12 (Fig. 2B). Immunostaining of *scid*-human PC-3 bone tumor tissue showed immunolocalization of CXCL12 protein to bone lining cells and PC-3 tumor cells in the bone marrow (Fig. 2C).

Bone Cell-Associated CXCL12 Stimulates CXCR4-Dependent Migration of Prostate Cancer Cells

To study the role of stromal cell-associated soluble CXCL12 in inducing chemomigration of PC-3 cells, we employed a transwell co-culture system where PC cells were cultured with either bone stromal cells or bone tissue conditioned medium. We showed that purified CXCL12, bone stromal cells, and bone tissue conditioned medium all stimulated chemomigration of PC-3 cells. Pretreatment with anti-CXCR4 antibody inhibited PC-3 cell migration towards stromal cells in a dose dependent fashion (Fig. 3A). Pre-treatment of PC-3 cells with anti-CXCR4 antibody similarly inhibited bone conditioned media-induced chemomigration (Fig. 3B). These data indicated that cancer cell migration in bone co-cultures is dependent on CXCL12/CXCR4 signaling.

CXCL12-Induced MMP-9 Gene Expression and Secretion by Prostate Cancer Cells

As described above, bone cells and bone tissue stimulated directional migration of PC cells. Previously, we demonstrated that interaction of stromal cells with cancer cells led to MMP-9 expression by cancer cells [29], and expansion of cancer cells in bone was associated with MMP-9 expression by cancer cells [25] and an increase in net enzymatic MMP-9 in bone tissues [27]. Herein, we hypothesized that bone-derived CXCL12 induces CXCR4 signaling in cancer cells leading to MMP-9 expression and motility. To this end, we stimulated PC cells with exogenous CXCL12. MMP-9 gene expression was induced in PC-3 and LNCaP cells but not in DU145 cells (Fig. 4A), correlating with the presence or absence of the CXCR4 receptor (Fig. 1B). Full-length MMP-9 gene promoter transfection studies with PC-3 cells showed that CXCL12 activated the MMP-9 gene promoter (Fig. 4B). Furthermore, CXCL12 treatment of PC-3 cells induced secretion of proMMP-9 protein into the conditioned medium in a dose-dependent fashion (Fig. 4C).

CXCL12-Mediated MMP-9 Expression and Chemoinvasion is Sensitive to PI3-Kinase and MEK Kinase Inhibitors

The PI3 kinase and MAP kinase pathways have been shown to be activated by CXCL12/CXCR4 interaction [23,37,38]. Pretreatment of PC-3 cells with either LY294002 (PI3 kinase inhibitor) or U0126 (MEK inhibitor) abrogated CXCL12-induced MMP-9 gene expression (Fig. 5A). Zymographic analysis of conditioned medium showed that CXCL12-induced pro-MMP-9 release was inhibited by pretreatment of PC-3

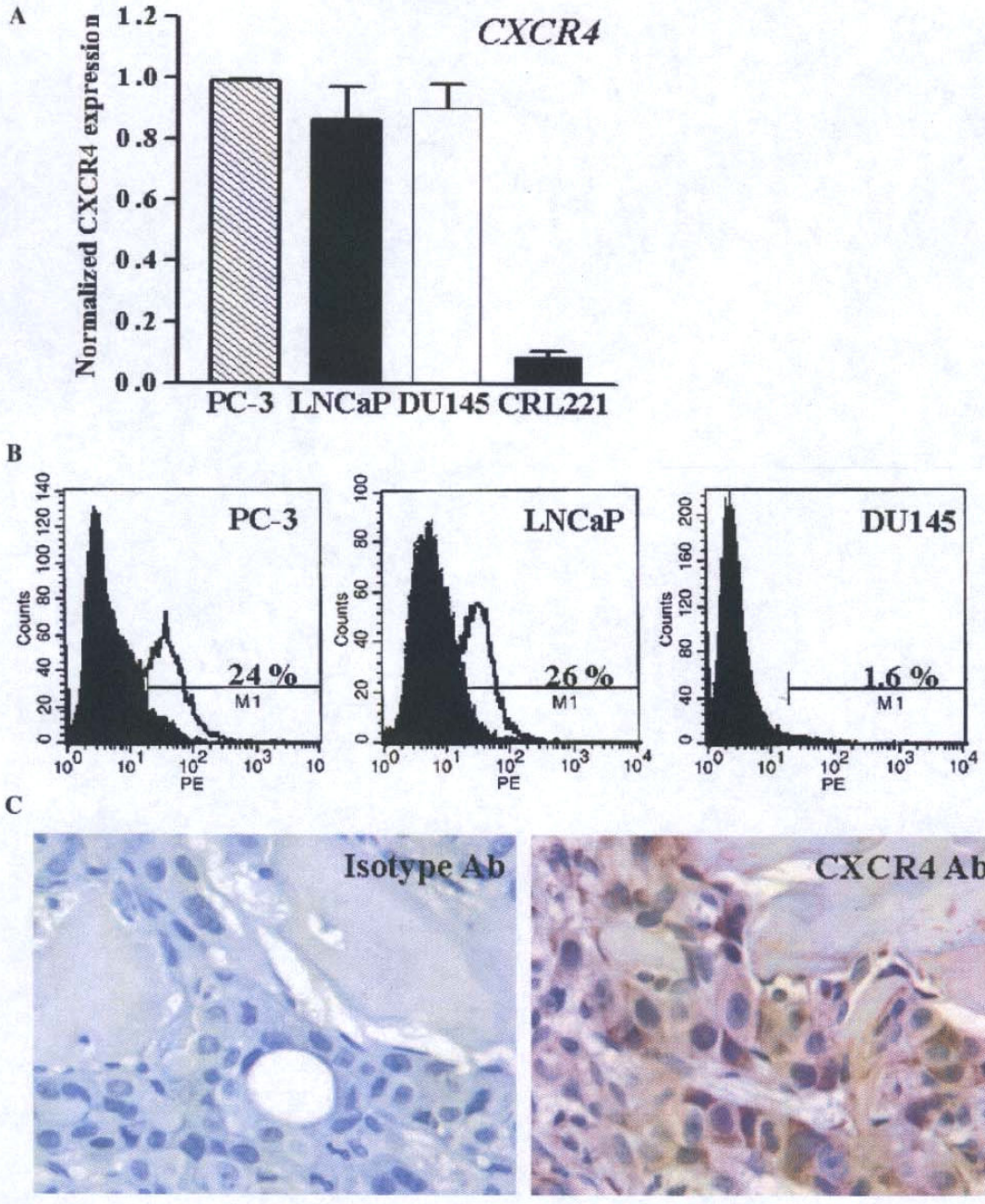


Fig. 1. CXCR4 expression in prostate cancer (PC) cells and benign prostate epithelial cells. **A**: Real time PCR analysis of mRNA isolated from three-cancer cell lines (PC-3, LNCaP, and DU145) and one immortalized benign epithelial line (CRL2201) using *CXCR4* gene primers. Gene expression levels were normalized first to GAPDH expression levels and then displayed relative to levels in PC-3 cells using the $2^{-\Delta\Delta Ct}$ method. The data represent mean ± SE of three independent real time PCR experiments. **B**: FACS analysis using a PE-conjugated anti-CXCR4 antibody and isotype-matched antibody control. **C**: Immunohistochemical analysis of a decalcified, formalin-fixed, paraffin embedded PC-3 bone tumor using an anti-CXCR4 antibody and isotype matched antibody control.

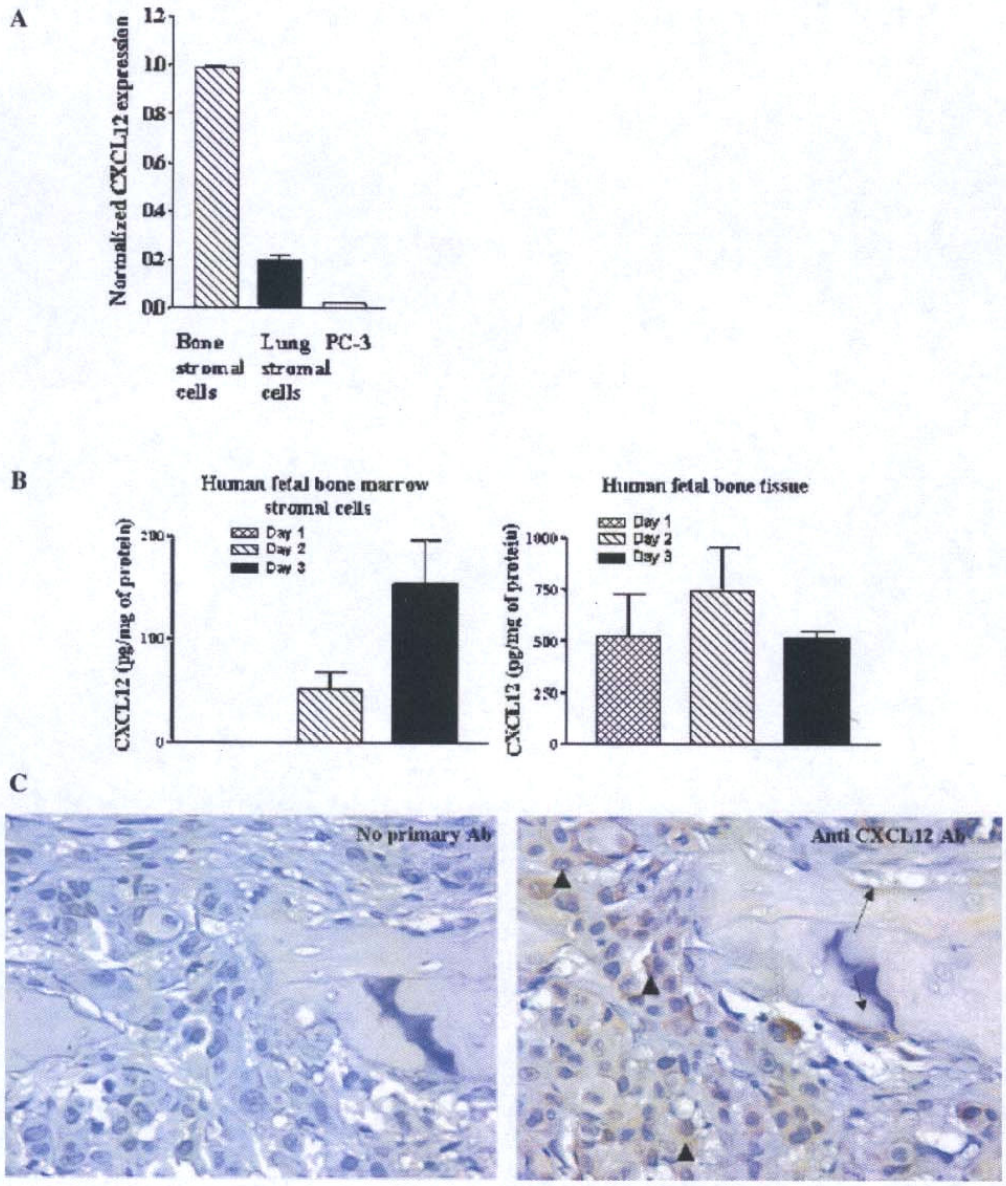


Fig. 2. CXCLI2 expression. **A**: Real time PCR analysis of mRNA isolated from human fetal bone stromal cells, human lung stromal cells, and PC3 cells using CXCLI2 gene primers. Gene expression levels were normalized first to GAPDH expression levels and then displayed relative to levels in human fetal bone stromal cells using the $2^{-\Delta\Delta Ct}$ method. The data represent mean ± SE of three independent real time PCR experiments. **B**: CXCLI2 release from human fetal bone stromal cells and human fetal bone tissues. ELISA was used to measure CXCLI2 protein levels relative to total protein levels in conditioned media on the first, second, and third day of culture. The data represent mean ± SE from three independent experiments using triplicate samples. **C**: Immunohistochemical analysis of a decalcified, formalin-fixed, paraffin embedded PC-3 bone tumor using an anti-CXCLI2 antibody. Arrow represents bone-lining cells and arrowhead represents cancer cells.

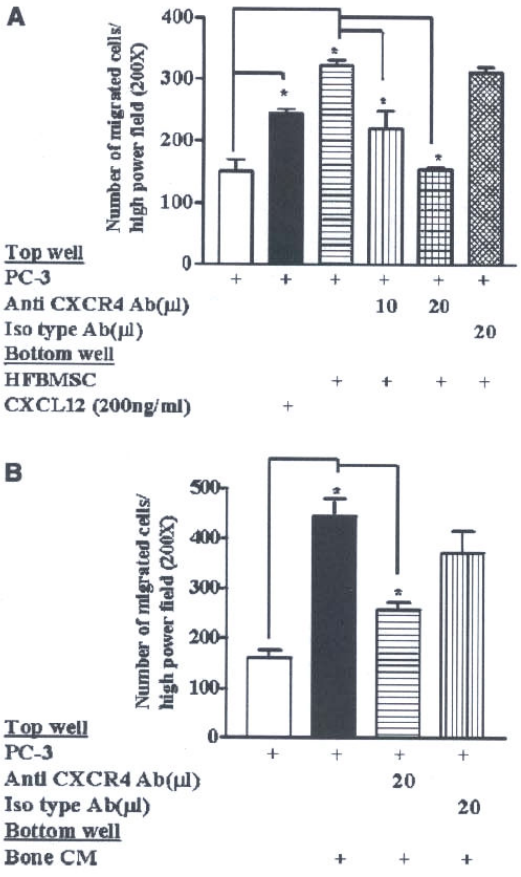


Fig. 3. Migration of PC-3 cells induced by bone is CXCR4-dependent. **A**: PC-3 cell migration was assessed in a transwell co-culture assay with human fetal bone stromal cells. PC-3 cells were seeded in upper chamber and human fetal bone stromal cells were seeded in bottom well. After 16 hr of incubation, the inserts were stained and number of migrated PC3 cells were scored. **B**: PC-3 cell migration was assessed in a similar fashion using conditioned medium from human fetal bone tissue as a chemo attractant. Experiments were performed in triplicate, and the data were analyzed using ANOVA. The tukey post-test was performed for pair-wise comparisons, and * represents P values ≤ 0.05.

cells with LY294002 and U0126 (Fig. 5B). One of the biological activities of cancer cell-derived MMPs are to facilitate invasion of the extracellular matrix [39,40]. Using matrigel coated cell culture inserts, we showed that pharmacological inhibition of either the PI3 kinase or MAP kinase pathway abolished CXCL12-induced chemoinvasion of PC-3 cells (Fig. 5C). LY294002 appeared to be more potent that U0126 in inhibiting CXCL12-induced MMP-9 gene and protein expression as well as chemoinvasion in PC cells.

CXCLI2-Induced Akt Phosphorylation in PC-3 cells

Published results show that CXCL12 activates Erk 1 and 2 [37], as well as Akt [38] in different PC cell lines [38]. Here, we found that CXCL12 stimulation led to activation of Akt in PC-3 cells (Fig. 6A), and this activation was sensitive to pretreatment of PC-3 cells with LY294002. These data suggested that Akt phosphorylation was mediated via the PI3 kinase pathway. Immunohistochemical analysis of SCID-human PC-3 bone tumors demonstrated specific staining for both Akt1 and $pAkt^{Ser473}$ in both cancer cells and multinucleated osteoclasts residing near bone trabeculae (Fig. 6B).

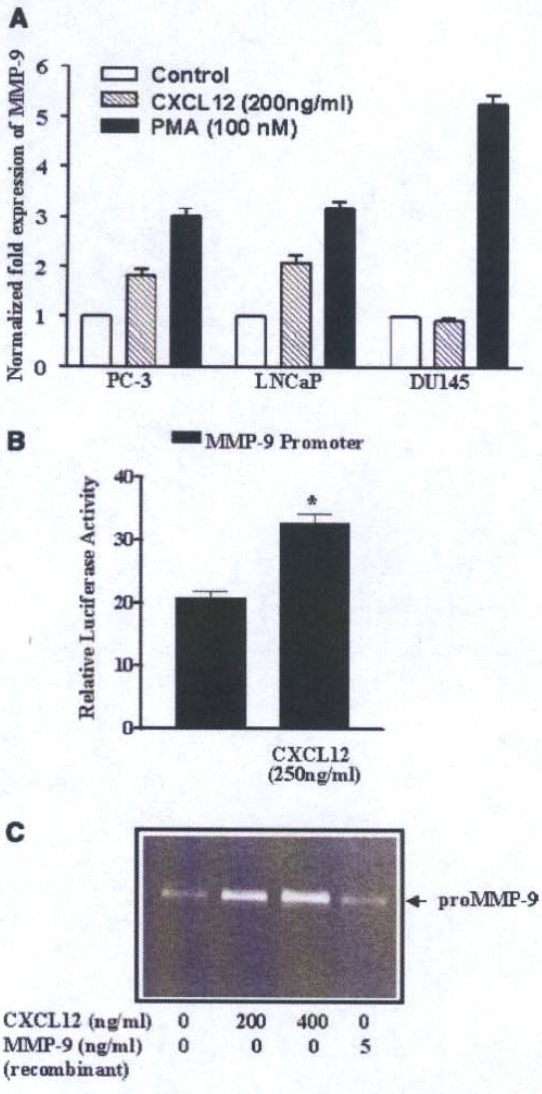
Akt1 Kinase Activation is Indispensable for CXCLI2 Induced MMP-9 Gene Expression, Release, Migration, and Invasion of PC-3 Cells

Previous studies showed that the three known isoforms of Akt were expressed in PC cells [31]. We used a sensitive real time PCR method to quantify the relative abundance of the various Akt isoforms in our cells (Fig. 7A). We found that the Akt1 gene is the most abundant isoform expressed in all the cell lines tested. To further understand the role CXCL12-induced activation of the PI3 kinase pathway in proMMP-9 secretion, cell migration, and invasion, we employed siRNA methodology to knock down Akt1. Western blot analysis of cells transiently transfected with Akt1 siRNA showed that Akt 1 protein expression was specifically down regulated in PC-3 cells (Fig. 7B). Furthermore, down regulation of the Akt1 isoform by siRNA diminished the overall levels of 473pSer-activated Akt (Fig. 7C), suggesting that CXCL12 signaling involves the Akt1 isoform in PC-3 cells. We then stimulated the Akt1 siRNA-transfected cells and control-transfected cell with CXCL12 and found that MMP-9 secretion was inhibited only in the Akt1 siRNA transfected cells (Fig. 7D). Furthermore, Akt1 siRNA transfection specifically inhibited CXCL12-induced migration (Fig. 7E) and invasion (Fig. 7F). Together, these data showed that Akt1 signaling was unequivocally involved in CXCL12-induced MMP-9 secretion, migration, and invasion by PC-3 cells.

CXCLI2/CXCR4 Interaction Leads to Activation of the NF-κB Response Element

Recent studies showed that activated Akt transduces signals via the cellular activation of NF-κB transcription factor [41,42]. Among the secreted MMPs, only MMP-9 has an NF-κB transcription factor binding

element in the promoter sequence [43], and we previously showed that PC-3 cells are capable of responding to external stimuli by activating NF-κB transcription factor activity [44]. Here we evaluated whether CXCL12/CXCR4 signaling can act via NF-κB. We transfected PC-3 cells with an NF-κB response element fused to a luciferase reporter construct. CXCL12 treatment of the transfected cells led to luciferase expression demonstrating activation of the NF-κB response element (Fig. 8). These data suggest that CXCL12/CXCR4-mediated PI3K/Akt activation leads to expression of NF-κB responsive genes.



CXCR4 Localization and Akt Phosphorylation Within Lipid Rafts in Prostate Cancer Cells

Lipid rafts are cell membrane microdomains that modulate oncogenic signal transduction by maintaining steady state levels of growth factor receptors [45,46]. To understand the role of CXCR4 signaling events during CXCL12-induced migration of cancer cells, we isolated rafts from PC3 cells using a sequential detergent solubilization technique [36]. As expected, lipid raft marker $G_i\alpha$-3 localized to the triton-insoluble portion of the plasma membrane in PC-3 cells and LNCaP cells, and caveolin localized to the triton-insoluble fraction of the plasma membrane in PC-3 cells (Fig. 9A), confirming the successful isolation of rafts. LNCaP cells are known not to express caveolin [47]. Western blot analyses of lipid raft and non-raft fractions of PC-3 and LNCaP cells showed that CXCR4 was enriched in the lipid raft fraction (Fig. 9B). Phosphorylated Akt also differentially localized to the various fractions. Ser 473 phosphorylated Akt species localized specifically to non-raft fractions whereas Thr 308 phosphorylated Akt localized exclusively to raft fractions (Fig. 9C). The phosphorylation of Akt was observed as early as 5 min and maximal at 15 min upon CXCL12 treatment of PC-3 cells (Fig. 9C and D). At present it is not known why Ser 473 and Thr 308 phoshporylated Akt molecules differentially localized to non-raft and raft regions on plasma membrane and further experiments are in progress to understand the significance of Akt activation in lipid raft regions of the plasma membrane.

DISCUSSION

Most patients with metastatic PC have skeletal involvement. A better understanding the factors

Fig. 4. CXCL12-induced MMP-9 gene expression and release from PC cells. **A**: Real time PCR analysis of MMP-9 gene expression in PC-3, LNCaP, and DU145 cells treated with CXCL12 (200 ng/ml for 24 hr). MMP-9 gene expression was normalized to GAPDH gene expression, and fold-induction over untreated cells is shown. Maximal MMP-9 gene expression induced by PMA (100 nM) is shown as a positive control. The data are presented as mean ± SE of three independent real time PCR experiments. **B**: CXCL12-induced MMP-9 promoter activation in PC-3 cells. PC-3 cells were transfected with a plasmid containing the MMP-9 promoter fused with the luciferase gene (pGL3-MMP-9). The same cells were co-transfected with a plasmid containing *Renilla* luciferase fused with CMV (pRL-CMV) as a transfection control. PC-3 cells were treated with CXCL12, and luciferase activity was measured and normalized to *Renilla* luciferase activity. Fold induction of MMP-9 promoter activity is shown in PC-3 cells treated with CXCL12 over untreated cells. **C**: Gelatin zymographic analysis of conditioned medium from PC-3 cells treated with CXCL12 (100, 200, 400 ng/ml) for 24 hr. Recombinant proMMP-9 was used as a marker.

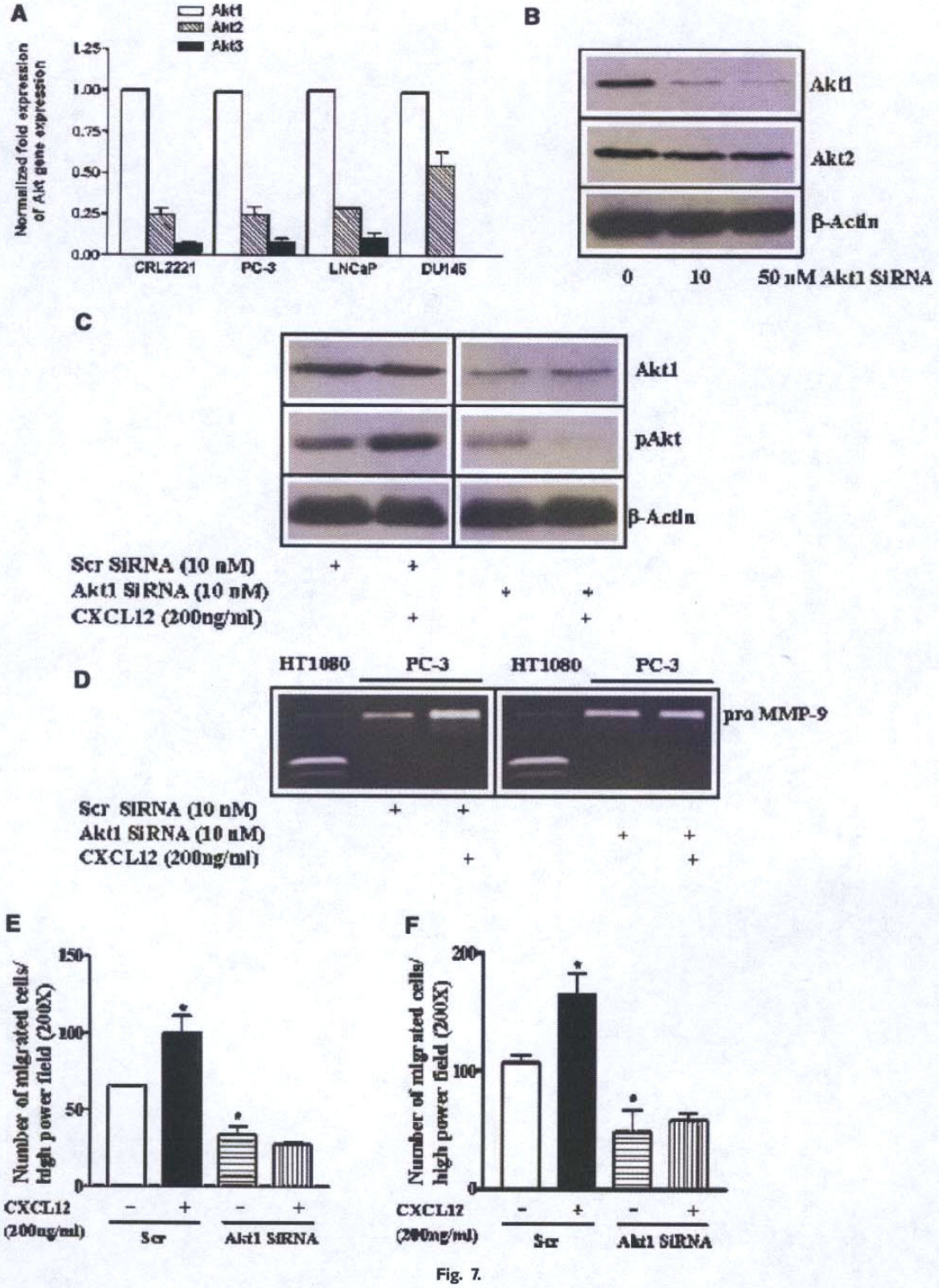

A
Akt1
Akt2
Akt3
Normalized fold expression of Akt gene expression
1.25
1.00
0.75
0.50
0.25
0.00
CRL2221
PC-3
LNCaP
DU145
B
Akt1
Akt2
β-Actin
0
10
50 nM Akt1 SiRNA
C
Akt1
pAkt
β-Actin
Scr SiRNA (10 nM)
Akt1 SiRNA (10 nM)
CXCL12 (200ng/ml)
D
HT1080
PC-3
HT1080
PC-3
pro MMP-9
Scr SiRNA (10 nM)
Akt1 SiRNA (10 nM)
CXCL12 (200ng/ml)
E
Number of migrated cells/ high power field (200X)
150
100
50
0
CXCL12 (200ng/ml)
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Fig. 7.

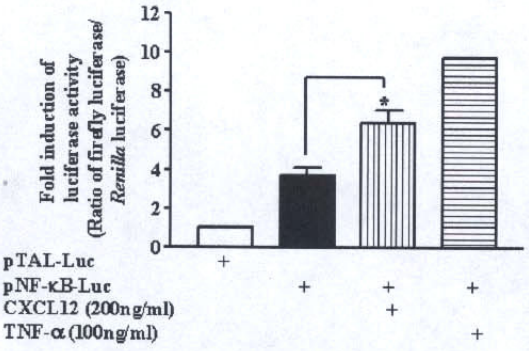

Fig. 8. CXCLI2 stimulates expression of the NF-κB response element. PC-3 cells were transfected with empty luciferase vector, pTAL-Luc vector, or NF-κB-Luc vector. Transfected cells were treated with either CXCLI2 or TNF-α, and normalized luciferase activities are shown. TNF-α treatment used as a positive control, which has shown to activate NF-κB transcription factor. The transfections were performed in triplicate, and the data were analyzed using the Student *t*-test. Data are expressed as mean ± SE, and * represents *P* values ≤0.05.

A distinguishing feature of bone metastasis is enhanced turnover of the extracellular matrix. Clinically, this is usually recognized radiographically, although signs of bone formation and/or degradation can be detected by other methods. Previously, we and others showed that pharmacologic broad spectrum inhibition of MMP enzymatic activity blocks bone matrix turnover as well as tumor cell proliferation and expansion of skeletal metastatic deposits [25,26]. Using a direct tissue assay of net enzymatic activity in an animal model of bone metastasis, we recently found a selective increase in net MMP-9 activity in bone during expansion of the metastatic deposit [27]. The MMP-9 protein immunolocalized to cancer cells and osteoclasts. Herein, we explored the hypothesis that microenvironmental factors such as CXCL12 induce expression and secretion of MMP-9 by PC cells, thereby linking the processes of chemoattraction, bone matrix turnover, and tumor cell proliferation in bone.

In this report, we showed that *CXCR4* gene expression was higher in PC cells than in immortalized benign prostate epithelial cells (Fig. 1A). These data are consistent with prior findings demonstrating expression of CXCR4 by PC cells [30,37,52]. In culture, we found that cell surface expression of CXCR4 protein was confined to a fraction of the cells (Fig. 1B). However, in the in vivo bone microenvironment, all of the cancer cells appeared to express CXCR4 (Fig. 1C). These data support the notion that microenvironmental factors stimulate expression of the receptor [11,13]. With regard to the ligand, we found high levels of CXCL12 gene expression in bone and lung stromal cells (Fig. 2A). Since our in vivo model of skeletal metastasis depends on the use fetal bone tissue, we examined human fetal bone stromal cells and bone tissue for secretion of CXCL12 protein. We found that isolated fetal bone stromal cells and intact fetal bone tissue secreted detectable amounts of CXCL12 (Fig. 2B). These data are consistent with the finding that osteoblasts [37,50] and stromal cells [53] express CXCL12. Interestingly, CXCL12 immunolocalized to cancer cells as well as to bone cells in the tissue (Fig. 2C), consistent with the idea that CXCL12 can act in an autocrine fashion [30]. CXCL12 can also act in a paracrine fashion; in fact, this chemokine has been shown to induce MMP-9 gene expression in osteoclasts [24]. Together, these data suggest that cancer cells respond to the bone environment by increasing expression of both chemokines and their receptors. In our system, the CXCL12/CXCR4 pathway was functionally significant; we showed that intact bone and isolated bone stromal cells stimulated a potent chemomigratory response that was dependent on CXCL12/CXCR4 (Fig. 3).

The CXCL12/CXCR4 pathway was first identified to be functionally significant in the homing or mobilization of hematopoietic stem cells (reviewed in [22]). The targeting of hematopoietic cells to new organ microenvironments is enhanced by the expression of proteases [54], and previous data demonstrated that CXCL12 binding to CXCR4 stimulates expression of protease(s) in several types of stem cells [55] and immune cells [56,57]. Since MMP-9 activity may be important for the establishment of bone metastases [27], we examined the influence of CXCL12/CXCR4 signaling on MMP-9 gene expression and protein secretion by tumor cells. We found that CXCL12 induced MMP-9 gene expression and protein secretion (Fig. 4), and this occurred only in cells expressing CXCR4. These data are similar to recent findings that

Fig. 7. Akt1 oncogene is essential for CXCLI2-induced MMP-9 gene expression, cell migration, and invasion. **A**: Real time PCR was used to assess gene expression of three isoforms of Akt in a panel of cultured cell lines. **B**: Immunoblot analysis demonstrating successful knock down of Akt1 expression using a siRNA approach. **C**: PC-3 cells transfected with either scrambled (scr) siRNA or Akt1 siRNA were treated with CXCLI2 for 15 min. Total cellular proteins were immunoblotted with anti Akt1, anti pSer473, and anti β-actin antibodies. **D**: PC-3 cells transfected with either scrambled siRNA or Akt1 siRNA were treated with CXCLI2 for 16 hr. Conditioned media were subjected to gelatin zymography. **E**: Migration and (**F**) invasion of PC-3 cells transfected with either scrambled siRNA or Akt1 siRNA and treated with 200 ng/ml of CXCLI2. Transfections were performed in triplicate for all the experiments, and data were analyzed using ANOVA and * represents *P* values ≤0.05.

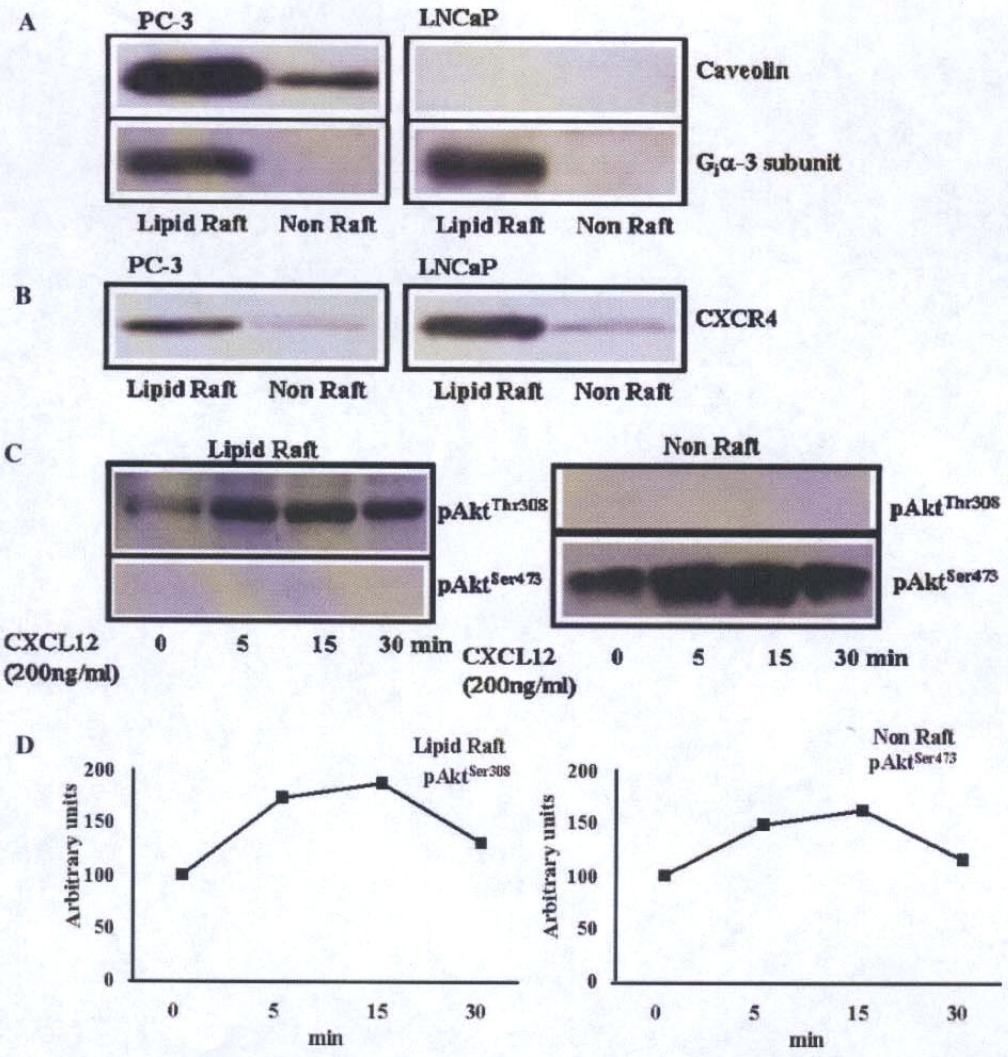

Fig. 9. CXCR4 localized to lipid rafts. **A**: Lipid raft fractions were isolated from PC cells using a successive detergent solubilization technique. The raft and non-raft fractions were immunoblotted with anti caveolin and anti $G_i\alpha$-3 subunit antibodies to confirm successful isolation of rafts. **B**: Immunoblot showing localization of CXCR4 to lipid raft fractions. **C**: PC-3 cells were treated with 200 ng/ml CXCL12 for 5, 10, and 15 min. Total proteins from the raft and non-raft fractions were immunoblotted with anti pSer473 Akt and anti pThr308 Akt antibodies. **D**: Pixel intensities in the immunoblot of figure C were quantified.

CXCL12 induces both MMP-2 and MMP-9 secretion in breast cancer cells [58] and hematopoietic progenitor cells [59–61], MMP-9 in osteoclasts [24], MMP-2 in rhabdosarcoma cells [62], and several MMPs in PC cells [52].

Pharmacological inhibition of the PI3 kinase pathway and the MAP kinase pathway abrogated CXCL12-induced MMP-9 gene expression and protein secretion, suggesting that MMP-9 production is a functional downstream target of both of these signaling pathways

(Fig. 5A and B). The PI3 kinase pathway has been shown to be involved in the chemotaxis of leukocytes by mediating actin polymerization and cytoskeletal changes leading to lamellipodium formation [63]. The MAP kinase pathway, involving p44/42, has been shown to be involved in proliferation, survival, and migration of tumor cells [21,30]. Our data suggest a metastasis-related function for the PI3 kinase and MAP kinase pathways wherein CXCR4 activation leads to expression and secretion of MMP-9. These data are consistent with a recent report [52] implicating the CXCL12/CXCR4 axis in metalloprotease expression by PC cells.

The literature suggests that the p110γ isoform of PI3-kinase is an upstream activator of the RAF–MEK–ERK pathway (reviewed in Ref. [64]). In addition, Akt has been shown to phosphorylate RAF, which is an upstream activator of MEK–ERK pathway [65]. Using pharmacologic inhibitors, we found that PI3 Kinase activity had a greater effect on MMP-9 expression than MAPK activity. Together, these data suggest that PI3 kinase may be upstream of MAPK in the CXCL12/CXCR4 signaling pathway, at least in PC3 cells.

We decided to study the role of the Akt protein in CXCL12/CXCR4 signaling because Akt activation has been shown to be dependent on PI3 kinase [66], and we found that CXCL12 stimulation induced PI3 kinase-dependent Akt activation in vitro and in vivo (Fig. 6A and B). We utilized siRNA methodology to specifically down regulate Akt1, the most predominantly expressed isoform in PC-3 cells. Silencing of Akt1 inhibited Akt activation and prevented CXCL12-induced MMP-9 secretion, cell migration, and invasion. Together, these data demonstrated that Akt phosphorylation is indispensable for CXCL12-mediated MMP-9 expression, as well cancer cell migration and invasion (Fig. 7).

As mentioned above, we found previously that MMP-9 may be more important than other MMPs in the establishment of bone metastases [27]. Since the MMP-9 gene is the only MMP gene with an NF-κB response element in its promoter [43], we examined the potential influence of CXCL12 signaling on NF-κB activity. We found that stimulation of cancer cells with CXCL12 induced activity of the transfected NF-κB response element (Fig. 8), suggesting that exposure of cancer cells to bone may lead to expression of a variety of NF-κB-responsive genes, including MMP-9.

Lipid rafts are specific microdomains in cell membranes that have been implicated as the physical sites for cross-talk between cell surface signaling molecules and their regulators [46]; rafts thus function as regulatory platforms for oncogenic signal transduction [45]. Rafts are enriched with several GPI-anchored proteins, non-receptor tyrosine kinases and G-protein subunits, all of which function as downstream modulators of seven-transmembrane receptors such as CXCR4. We showed that CXCR4 was associated with lipid rafts in PC cells, and CXCL12 initiated the activation of Akt kinase through lipid raft-associated CXCR4. Similarly, CXCR4 has been shown to be associated with lipid rafts of migratory leukocytes [67] and stem cells [61]. The integrity of lipid rafts, and association of CXCR4 to the raft shown to be required for the chemotactic signaling pathways of the T cells [68] and stem cells [68]. Together, these data suggest that CXCL12-induced signaling originates from the lipid raft signal transduction machinery. Interestingly, we observed differential localization of phosphorylated Akt molecules in lipid rafts and non-lipid raft fractions of PC cells. Published reports show that PDK1 phosphorylates T308 of Akt 1 and DNA-PK phosphorylates S473 of Akt [69]. Both of these upstream kinases were localized to lipid rafts of the plasma membrane during agonist stimulation and, both kinases phosphorylate Akt. Additional studies are in progress to delineate molecular mechanisms of CXCR4-mediated Akt activation in PC cells.

In summary, we demonstrated a novel role for CXCL12/CXCR4 interactions in PC (Fig. 10). Binding of CXCL12 to CXCR4 leads to expression and secretion of

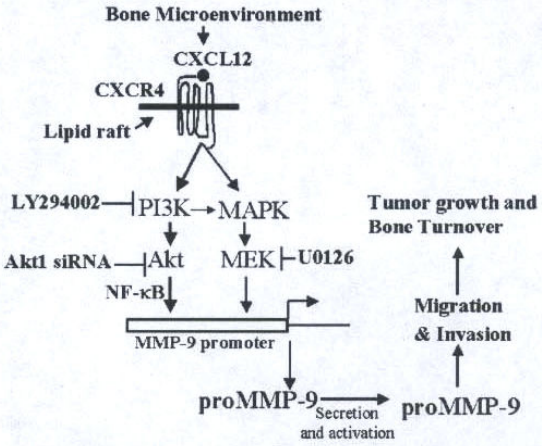

Fig. 10. Schematic representation of CXCL12-induced CXCR4 activation in cancer cells in the bone microenvironment. Exposure of PC cells to the bone microenvironment leads to binding of the bone-derived CXCL12 chemokine to the CXCR4 receptor in lipid rafts on the surface of cancer cells. This receptor–ligand interaction activates both the PI3 kinase and the MAP kinase pathways leading ultimately to Akt1 activation, NF-κB transcription factor activation, MMP-9 gene expression, MMP-9 protein release, and cellular migration and invasion. These findings link the processes of chemo attraction to MMP-9-mediated invasion, tumor growth, and bone remodeling.

MMP-9 via activation of both the PI3 kinase and MAP kinase pathways. Akt1, in particular, appears to be necessary for MMP-9 production and cell migration. Cell signaling appears to originate in lipid rafts, and activation of NF-κB likely leads to expression of a variety of other genes. Activation of CXCL12/CXCR4 cell signaling may thus serve as a link between chemomigration/invasion of cancer cells and cancer cell-induced bone matrix turnover and subsequent expansion of the metastatic deposit.

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